THE PERIODONTAL SYNDROME IN THE RICE RAT (ORYZOMYS PALUSTRIS). THE NATURAL PROGRESS OF DISEASE AND THE EFFECTS OF DICHLOROMETHYLENE DIPHOSPHONATE ON DISEASE PROGRESS

by

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ABSTRACT

Several morphometric and cellular parameters were studied in the rice rat (Oryzomys palustris). When fed a soft, high carbohydrate diet, a severe periodontal disease occurred, with significant alterations in the morphometric and cellular endpoints observed. Weaned animals were placed on a high carbohydrate diet for periods of 6, 12 or 18 weeks. There was a linear, rapid loss of bone by 18 weeks, approaching a 75% loss of original bone. Vascular spaces decreased as the remaining connective tissue became fibrotic in character. The percentage of the interdental test site which was destroyed by periodontal disease increased dramatically over the time of the experiment. The numbers of fibroblasts per mm of bone surface increased slightly at the 18 week period; osteoblasts were unchanged at any period. The numbers of osteoclast nuclei rose dramatically by 12 weeks, and these cell nuclei remained at increased levels at 18 weeks. Also, the numbers of inflammatory cells residing at the bone surface increased greatly by 18 weeks time. Finally, the numbers of ^{3}H-TdR labeled periodontal ligament (PDL) fibroblasts increased significantly at both 12 and 18 weeks time. These cellular changes and their relation to the bone loss due to periodontal disease are discussed.

The effects of a diphosphonate, dichloromethylene diphosphonate (Cl_2MDP) were also studied, using the rice rat as a model of periodontal bone loss. Cl_2MDP was given in daily subcutaneous injections

at dosages of 0 (control), 0.1, 1.0, or 10.0 mg/kg/day; these treatments were continued for periods of 6, 12, or 18 weeks. The amount of alveolar bone was increased over age matched controls at the 1.0 and 10.0 mg/kg/day doses at 6 weeks; at 12 and 18 weeks, all doses showed increases in bone over controls. Due to increased connective tissue fibrosis, the Cl_2MDP treated animals had fewer vascular spaces. Also, the amount of destroyed tissue in the interdental test site was increased in animals given 10.0 mg/kg/day of Cl_2MDP. The number of fibroblasts per mm of bone surface decreased slightly at 6 weeks, but was otherwise not different from controls, in treated animals at 12 and 18 weeks. Numbers of osteoblasts decreased greatly at all doses, at both 12 and 18 weeks time. There were no significant differences between treated and control animals in the number of osteoclast nuclei per mm bone surface. Also, the numbers of inflammatory cells residing at the bone surface increased at all time periods in the 10.0 mg/kg/day dose group. Finally, the proliferative activity of PDL fibroblasts decreased dramatically at all time periods in the 10.0 mg/kg/day dose group. The response of the proximal tibia to Cl_2MDP was compared to the alveolar bone response in these animals.

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PART I

THE PROGRESS OF THE PERIODONTAL SYNDROME IN THE RICE RAT

(Oryzomys palustris)

INTRODUCTION

The use of animal models in periodontal research has aided greatly in the elucidation of mechanisms and progression of periodontal destruction. The rice rat (Oryzomys palustris) has been shown to be an effective model for the study of periodontal disease (Gupta & Shaw 1955, Gupta & Shaw 1956a). In early reports, the pathology of the periodontium was described, and it was observed that wild strains of the animal would contact varying degrees of a periodontal syndrome, first seen at weaning, and progressing with age. The lesions were similar in location and appearance to those seen in man. Later, it was demonstrated that the periodontal lesions could be accentuated greatly by feeding the animals a diet which was high in sucrose (Auskaps, Gupta, & Shaw 1957, Shaw & Griffith 1961, Shaw 1965a, Shaw, Krumins & Gibbons 1967). It was also found that the presence of casein in the diet increased the severity of the periodontal syndrome (Shaw 1966). With a standard diet (Harvard 700 diet), the progress of the disease could be accelerated in test animals. In the course of several studies, it was discovered that two strains of rice rat, syndrome-prone and syndrome-resistant, could be bred. Despite long periods of feeding the Harvard 700 diet to syndrome-resistant animals, little or no periodontal destruction could be noted (Dick & Shaw 1966).

It has been well established that the periodontal destruction in the rice rat could be lessened by administration of antibiotics,

either as dietary supplements (Gupta, Auskaps & Shaw 1957, Shaw, Griffiths & Auskaps 1961, Shaw 1965b, Shaw and Ivimey 1973) or as daily oral swabbings (Leonard, Horton & Mandel 1978). This effect of antibiotics on periodontal lesions strongly implied that bacteria might play a major role in the development in this disease in the rice rat. Studies which surveyed and quantitated the bacterial flora of the rice rat (Macdonald, Socransky & Sawyer 1959, Socransky, Macdonald & Sawyer 1960) indicated that a strain of enterococcus might be the causative agent. Transmissibility of the periodontal syndrome was shown by using fecal paste obtained from diseased animals to infect animals from the syndrome-resistant strain, and producing disease in these resistant animals (Dick & Shaw 1966), further indicating the pivotal role of bacteria in the periodontal syndrome of the rice rat.

Histopathological changes were described in the initial reports of periodontal disease in the rice rat (Gupta & Shaw 1956b, Gupta & Shaw 1960), and later in more detail for prone and resistant animals, and animals given antibiotics (Mulvihill et al. 1967). As earlier demonstrated grossly, only the syndrome prone rats without antibiotics developed severe periodontal disease.

Periodontal lesions in syndrome prone animals were largely absent up to five weeks of age, in animals fed the Harvard 700 diet since weaning. By seven weeks, the first lesions became evident. There was apical migration of the junctional epithelium, with pocket formation and areas of cementum and alveolar bone resorption. By fifteen weeks of age, severe periodontal breakdown had occurred in the molars, with marked gingival recession and alveolar bone loss. The authors concluded that the periodontal syndrome in rice rats is

similar in many aspects to the disease in man. Recently, a study of the progress of periodontal disease in the rice rats was performed, using histochemical methods for cell identification (Leonard & Swing 1977). Early inflammatory infiltrate was composed of neutrophils and monocytes, and was alkaline phosphatase positive. At later times, an acid phosphatase positive infiltrate was observed in the epithelium and periodontal ligament. The authors noted an increase in osteoclast numbers against the bone surface in animals fed a high carbohydrate diet for more than 100 days.

The purpose of this study is to quantitate several aspects of the progression of periodontal disease in the rice rat. The general objectives of the study are to employ 1) morphometric techniques to observe changes in the areas of component tissues of the interdental periodontium, 2) cell population counts, to detect any changes in numbers of cell types present at the bone surface as the disease progresses, and 3) tritiated thymidine labeling of cells, to observe the proliferative activity of cells at the bone surface. It was hypothesized that changes in the above parameters would occur in a uniform manner as the disease progressed, and that these results would yield information as to the mechanisms of bone loss in the rice rat.

MATERIALS AND METHODS

Experimental Design

This experiment utilized 29 growing rice rats (Oryzomys palustris) of both sexes, selected from our breeding colony as periodontal syndrome "prone" animals.* The animals were incorporated into the experiment at weaning (21 days of age); the experimental course was staggered as new weanlings became available from our breeding colony.

At weaning, the animals were housed in individual stainless steel wire bottomed cages (4"x5"x7") and given tap water and Harvard 700 diet (Table 1) ad libitum. This high carbohydrate diet has produced a severe periodontal breakdown in our animals. All animals were raised and maintained under strict environmental conditions which included a 14 hour light, 10 hour dark cycle (light from 6:00 a.m.-8:00 p.m.), room temperature of 24 ± 1°C, and a controlled humidity of 35% - 45% (Shaw, 1976).

Animals were given the high carbohydrate diet for periods of 6, 12 or 18 weeks after weaning. Previous studies had shown that rice rats would develop a slight amount of disease by 6 weeks treatment, and a severe periodontal disease by 15 weeks of treatment (Mulvihill et al. 1967). There were at least 6 animals per time period sampled. All animals received a single daily subcutaneous injection of 0.9%

*We gratefully acknowledge the gift of original rice rat breeders from Dr. James H. Shaw, Harvard School of Dental Medicine, Boston, Mass. 02115.

TABLE 1.

CONTENTS OF HARVARD RATION 700 DIET

Sucrose, granulated	660 g.
Casein, purified high nitrogen	240 g.
Corn oil	50 g. (54 ml.)
Mineral Salt Mixture TD 77333 (Shaw))	40 g.
Liver powder	40 g.
Vitamin fortification mix	10.4 g

Supplies: Casein and Liver Powder; ICN Pharmaceuticals, Cleveland, Ohio. Corn Oil; Mazola, Best Foods, Div. of CPC, Englewood Cliffs, N.J. Mineral Salt Mixture and Vitamin Fortification Mix; Teklad Test Diets, Madison, Wisc.

sodium chloride solution (Bacteriostatic Saline, Abbott Laboratories, N. Chicago, Ill.) in the amount of 1.0 ml per 100 g. body weight. These injected animals served as controls in a dose response study of a diphosphonate as a therapeutic agent. The results of that study were reported separately. Injections were continued for the duration of the treatment periods of 6, 12, or 18 weeks. All animals were weighed and injected daily at the same time of day (8:00--9:00 a.m.).

At one hour prior to autopsy, at the end of the assigned treatment periods, each animal was given a single subcutaneous injection of 0.5 µCi per g. body weight of tritiated thymidine [methyl-^{3}H] (^{3}H-TdR, specific activity = 23.0 Ci/mM; New England Nuclear, Boston, Mass.). All ^{3}H-TdR injections and subsequent autopsies were performed at the same time of day (10:00--11:00 a.m.) to avoid any diurnal variations.

Histological Techniques

All animals were sacrificed by either anesthetic overdose;

samples taken at autopsy included the mandible and tibial metaphyses. The tissues were immersed immediately in warmed (37°C) neutral phosphphate buffered 10% formalin solution, and fixed for 24 hours at room temperature. The tibial metaphyses were dehydrated and defatted, while the mandibular tissues were processed as described below.

The right mandibular halves were decalcified in 14% EDTA solution (pH = 7.2), then carefully trimmed under a dissecting microscope in a mesiodistal (dental parasagittal) plane, through the roots and apical foramina of the molar teeth. These tissue blocks were embedded in modified methyl methacrylate (Kimmel & Jee 1975), and sectioned in the mesiodistal plane. Sections of 3 μm thickness were taken in a semiserial manner, so that adjacent sections were separated by at least 12 μm of tissue space from each other. A motorized Jung Model K microtome (R. Jung, Heidelburg, W. Germany) was used to section all mandibular tissues. Some sections were dipped in Kodak NTB emulsion (Eastman Kodak, Rochester, N.Y.), diluted 1:1 with deionized water, for autoradiography. These sections were exposed in light tight containers at -18°C for 4 weeks, then developed for 3.5 minutes with Kodak D-19 at 20°C, fixed and stained with hematoxylin and eosin. Other sections were prepared for histological studies by staining with hematoxylin and eosin.

Quantitative Methods

The area selected for study was the interdental area between the first and second mandibular molars, as this area was affected extensively with the periodontal syndrome. In addition, histological sections of equivalent orientation in this region were easily

reproducible. The area selected was bounded by the root surfaces of the first and second mandibular molars (M_1 and M_2), and lines drawn between the apical foramina of M_1 and M_2 (Figure 1).

Area based analyses

The area occupied by different tissues within this region was determined by point counting methods (Elias, Hennig & Schwartz 1971) using a 100 point eyepiece reticule (American Optical, Buffalo, N.Y.) at a magnification of 250x. Each interdental area studied contained approximately 0.7-1.0 mm^2; with a grid area of 0.16 mm^2 at this magnification, an average of 6 grid fields were counted per area. In total, 4 non-adjacent sections per animal were analyzed.

The four tissue components measured were: 1) bone, 2) soft tissue, including epithelium, subjacent connective tissue and periodontal ligament, 3) vascular spaces, being mostly thin walled veins, and 4) area formerly occupied by any of the above tissues, but now missing, presumably destroyed by the periodontal disease process ("destroyed tissue" space). Because all of the animals may not express the periodontal syndrome (Hattler et al. 1977), it was necessary to establish if the disease was present in each animal. The animal was considered to have disease if 1) observation of histologic sections revealed any pathological changes, and 2) if some tissue destruction had taken place in the interdental area at the longer sampling periods (2% destruction at 12 weeks, 5% destruction at 18 weeks). Thus, animals which were disease free at these long time periods were excluded from quantitative analysis.

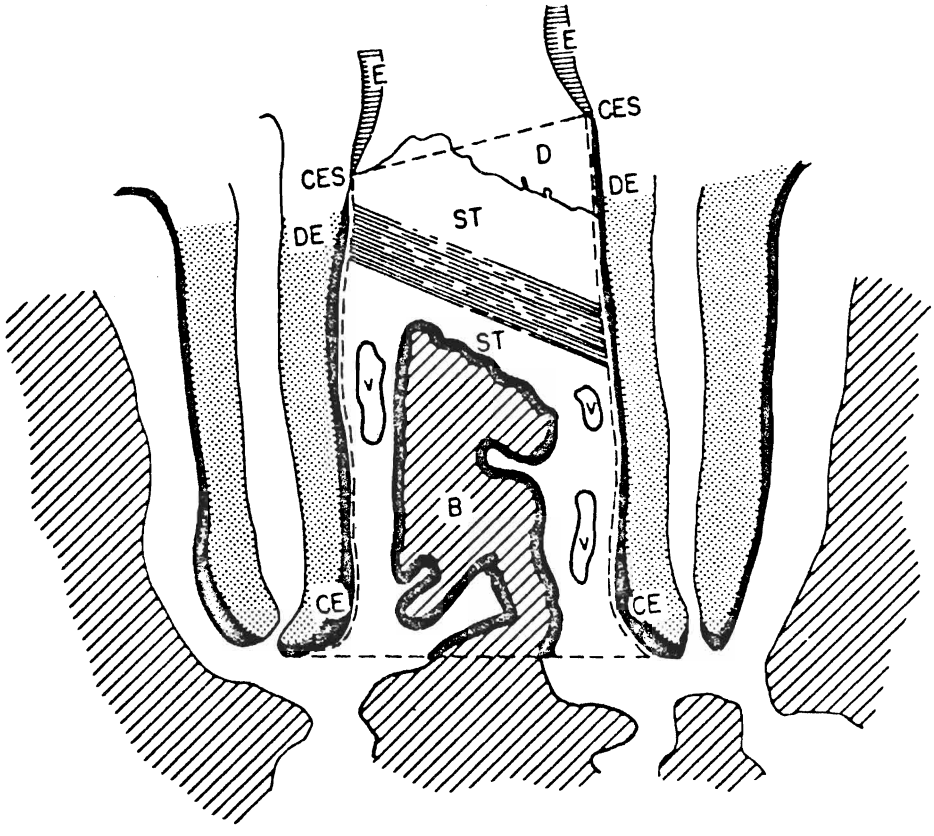


FIGURE 1. Diagram of interdental area studied in experiment. Dashed lines represent the boundaries of the test area. M = first mandibular molar, M + second mandibular molar, E = enamel, DE = dentin, CES = cementoenamel junction, CE = cementum, D = space formerly occupied by tissue, now destroyed, ST = soft tissue, including epithelium and subjacent connective tissue, V = vascular space, B = alveolar bone.

Cell population analyses

The site selected for study was the bone surface of the interdental alveolar bone between the first and second mandibular molars, as outlined in black (Figure 1). Along this surface and approximately 20 µm adjacent into the periodontal ligament (PDL), numbers of PDL fibroblasts, osteoblasts, osteoclast nuclei, and inflammatory cells were counted.

PDL fibroblasts were identified as spindle shaped cells with inconspicuous cytoplasm and a prominent nucleus with variable numbers of nucleoli. This cell type represents a morphologically similar pool of cells, including mature fibroblasts, as well as proliferating cells renewing the cell population of the periodontal ligament (Melcher & Eastoe 1969). This cell was the major type at or near the bone surface. Osteoblasts were identified as rounded or elliptical cells with basophilic cytoplasm, and eccentrically located nucleus, and a juxtanuclear clear zone corresponding to a functioning Golgi apparatus. Osteoclasts were seen as large, usually multinucleated cells, adjacent to the bone surface, often in a Howship's lacuna. These cells had a distinctive pale or slightly eosinophilic "foamy" cytoplasm, with nuclei often irregular in shape, having a prominent nucleolus. Inflammatory cells were identified as any of the cell types involved in either the acute or chronic inflammatory response. Possible cell types included polymorphonuclear neutrophils (PMN's), lymphocytes, plasma cells, monocytes and macrophages. The majority of inflammatory cells noted at the bone surface were PMN's, with monocytes or macrophages noted on occasions. Phase microscopy was employed for the identification of all cell types.

These cell populations were normalized by relating them to the length of the bone surface perimeter. Bone surface perimeter was determined by counting the intersections of the bone surface with the lines of an eyepiece ocular Merz grid (Merz & Schenk 1969), at a magnification of 625x. Grid lines were spaced at 0.027 mm intervals; approximately 10 grid areas were counted along the bone surface of a typical animal from the 6 week sampling time. The length of bone surface perimeter was determined by applying the formula:

$$\text{Perimeter} \quad I \times D \times \pi/2,$$

where I = numbers of intersections counted, D = distance between the eyepiece grid lines at 625x, and $\pi/2$ is a correction factor (Elias et al. 1971). There were two non adjacent sections per animal analyzed per group.

Proliferative activity of PDL fibroblasts

The fraction of PDL fibroblasts which were synthesizing DNA was determined by observation of numbers of cells incorporating tritiated thymidine (^{3}H-TdR) as determined by autoradiographs. The labeling index (LI) of the PDL fibroblast was defined as the number of ^{3}H-TdR labeled cells divided by the total number of cells in a given area. A cell was considered to be labeled if there were 4 or more silver grains directly over the nucleus; there was no significant background in the autoradiographs. In addition to the LI, the absolute numbers of labeled fibroblasts were normalized to the mm of bone surface perimeter.

Statistical Methods

All data were expressed as value ± Standard Error of the Mean (S.E.M.). The significance of group differences was established

initially by analysis of variance methods (Snedecor & Cochran 1967). If the analysis of variance showed significant changes between groups, significant differences between individual groups were found using Duncan's test of multiple comparisons (Duncan 1955), with revised tables (Harter 1960).

RESULTS

Observations

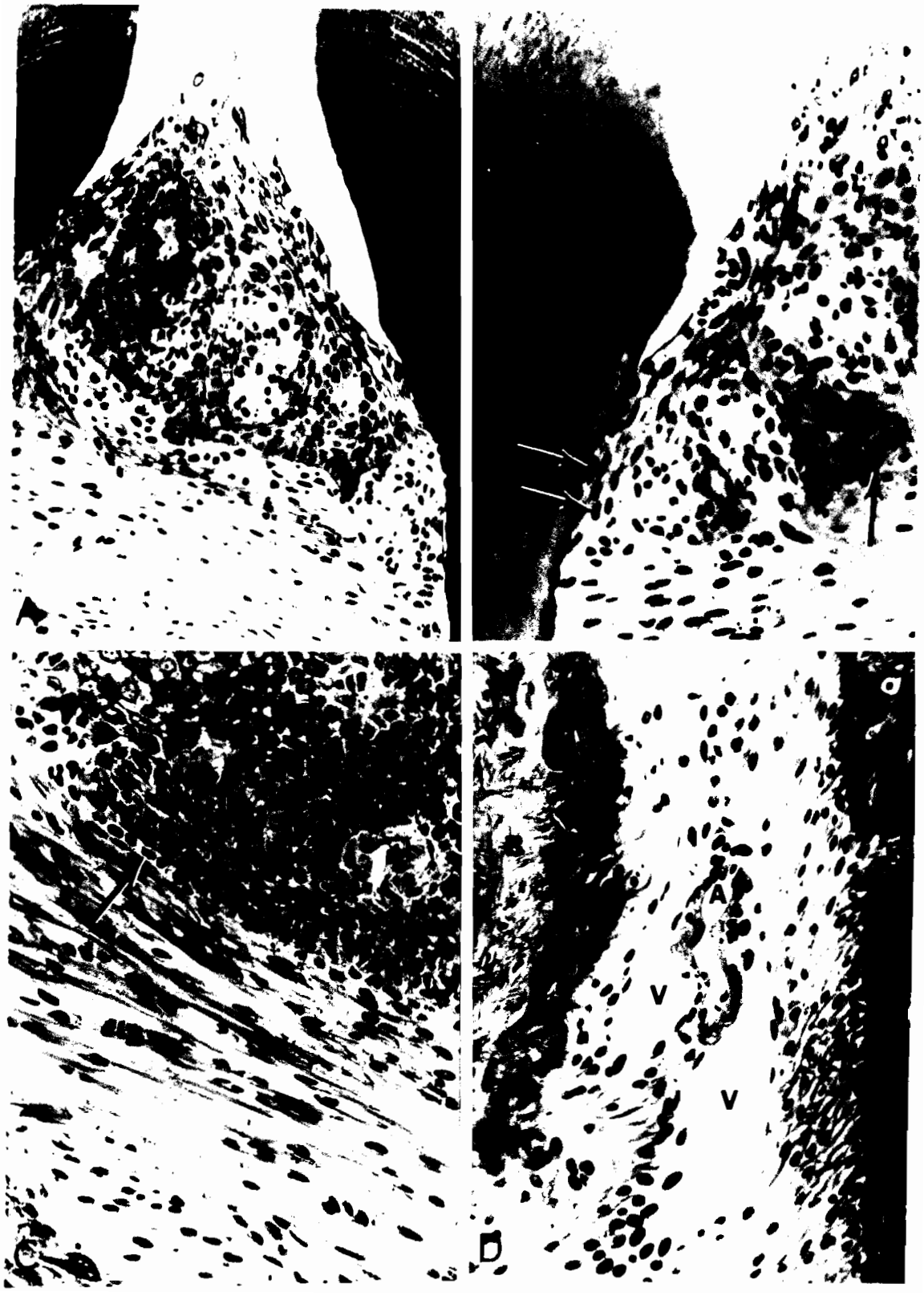
The periodontal syndrome of the rice rats progresses to severe levels within the time periods of the experiment. This progress will be described, at each time period, for the epithelium, subepithelial and periodontal ligament connective tissue, and alveolar bone, within the area studied.

After 6 weeks of the Harvard 700 diet, rice rats demonstrate signs of early periodontal disease. In this group of animals, there is a generalized infiltration of PMN's and lymphocytes within the epithelium (Figure 2A). The epithelial attachment is often disrupted, and some apical proliferation of epithelium is occurring (Figure 2B). Moderate accumulation of plaque can be seen on tooth or epithelial surfaces. At 6 weeks, there are relatively few transeptal inflammatory cells in the transeptal fibers or periodontal ligament, despite an often prominent epithelial infiltration (Figure 2C). The periodontal ligament has well arranged fiber bundles, and large vessels which occupy much of the space between the alveolar bone and cementum (Figure 2D).

The alveolar bone, at 6 weeks of high carbohydrate diet administration, is undergoing both formation and resorption in selected areas. Figure 3A demonstrates the overall view of the alveolar bone, showing the generally smooth contours of the bone that is adjacent to the periodontal ligament. The alveolar crest shows areas of active osteoblasts

FIGURE 2 - A-D

Decalcified 3 μm sections of the epithelium and subjacent connective tissue of rice rats after 6 weeks of Harvard 700 diet. (A) Interdental epithelium, with moderate infiltration of PMN's and lymphocytes within the tissue (Original magnification 125x). (B) Autoradiograph of epithelial attachment, with inflammation and early apical migration of the epithelium. Arrows note ^{3}H-TdR-labeled cells, asterisk demonstrates the cementoenamel junction (Original magnification, 250x). (C) Boundary between gingival epithelium and gingival transeptal fibers, with several inflammatory cells at this boundary and a few within the subjacent connective tissue (Original magnification, 250x). (D) Periodontal ligament, with large vascular spaces, composed of thin walled veins (V) and an artery (A) (Original magnification, 250x).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(Figure 3B), or both osteoblasts and osteoclasts (Figure 3C). Osteoblast layers are observed in the apical portions of mesial and distal bone surfaces. Prominent osteoclasts within Howship's lacunae can be seen in areas of the mesial half of the alveolar bone. These bone modeling patterns are consistent with the physiological distal drifting of rodent molar teeth (Dreyer 1967, Roberts 1975).

At 12 weeks of treatment, further degenerative changes are noted within the periodontium. The epithelium is heavily infiltrated with PMN's and lymphocytes, and the normal architecture of the epithelium is disrupted. Within the epithelium, areas of high proliferative activity can be seen (Figure 4A), especially in the area of the periodontal pocket (Figure 4B). Inflammatory cells may be found within the transeptal fibers and periodontal ligament. Macrophages with eosinophilic phagocytic vacuoles could be observed in this connective tissue (Figure 4C), or adjacent to the bone surface (Figure 4D).

The alveolar bone surface shows a scalloped, irregular pattern, with a decrease in the amount of bone present (Figure 5A). These scalloped areas often contain osteoclasts, blood vessels, and many fibroblasts (Figure 5B), in areas formerly occupied by alveolar bone. The PDL fibroblasts within these scalloped areas show a higher level of proliferative activity than adjacent periodontal ligament, as evidenced by numbers of ^{3}H-TdR labeled cells (Figure 5C). The alveolar crest also shows a high level of proliferative activity (Figure 5D). Osteoclasts can be observed along all bone surfaces at this point, as the normal modeling pattern observed at 6 weeks becomes disrupted. Many cement lines are present within the bone, as the normal osseous

FIGURE 3 - A-D

Decalcified 3 μm sections of alveolar bone and periodontal ligaments from rice rats treated for 6 weeks with Harvard 700 diet. (A) Low power photomicrography showing normal quantity of alveolar bone with smooth contours (Original magnification, 75x). (B) Alveolar crest and periodontal ligament with no inflammatory cells present and several active osteoblasts (arrows) lining the bone surface (Original magnification, 250x). (C) Alveolar crest demonstrating the presence of both osteoblasts (single arrows) and an osteoclast in this region (Original magnification, 625x). (D) Mesial aspect of alveolar bone demonstrating osteoclasts (arrows) with Howship's lacunae (Original magnification, 250x).

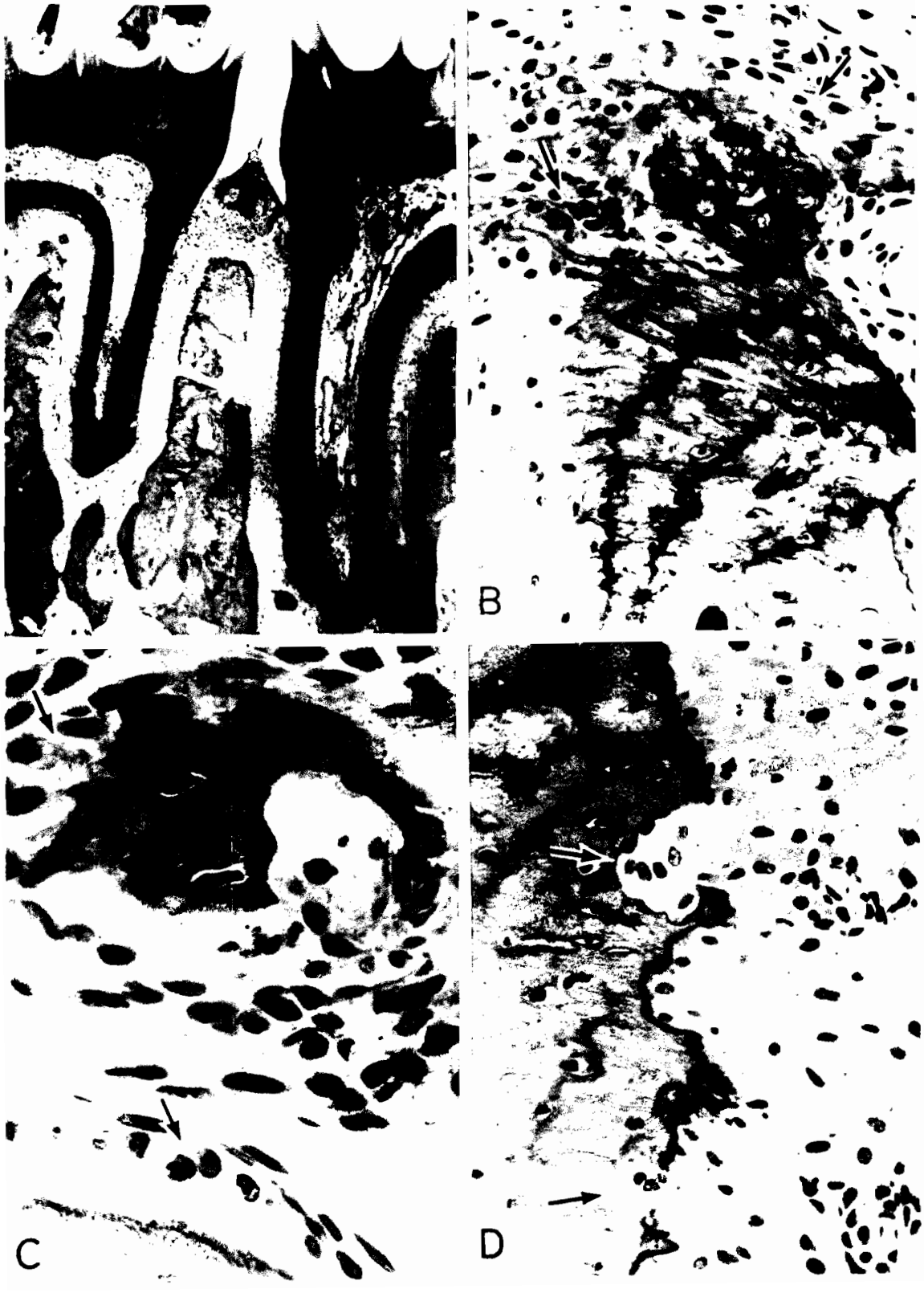
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FIGURE 4 - A-D

Decalcified 3 μm sections of epithelium and subjacent connective tissue of rice rats treated with Harvard 700 diet for 12 weeks. (A) Autoradiographs of diseased epithelium, showing concentration of ^{3}H-TdR-labeled epithelial cells (Original magnification, 250x). (B) Autoradiograph of epithelial attachment in diseased animal at 12 weeks. Note the presence of several ^{3}H-TdR labeled cells (arrows) and impacted hair (asterisk) within the epithelium (Original magnification, 125x). (C) Transeptal fibers of diseased animal with a marked influx of macrophages (arrows) with deeply staining phagocytic vacuoles (Original magnification, 500x). (D) Adjacent areas to Figure 3C, showing macrophage adjacent to the bone surface, with a phagocytosed PMN within the cell (arrow) (Original magnification, 625x).

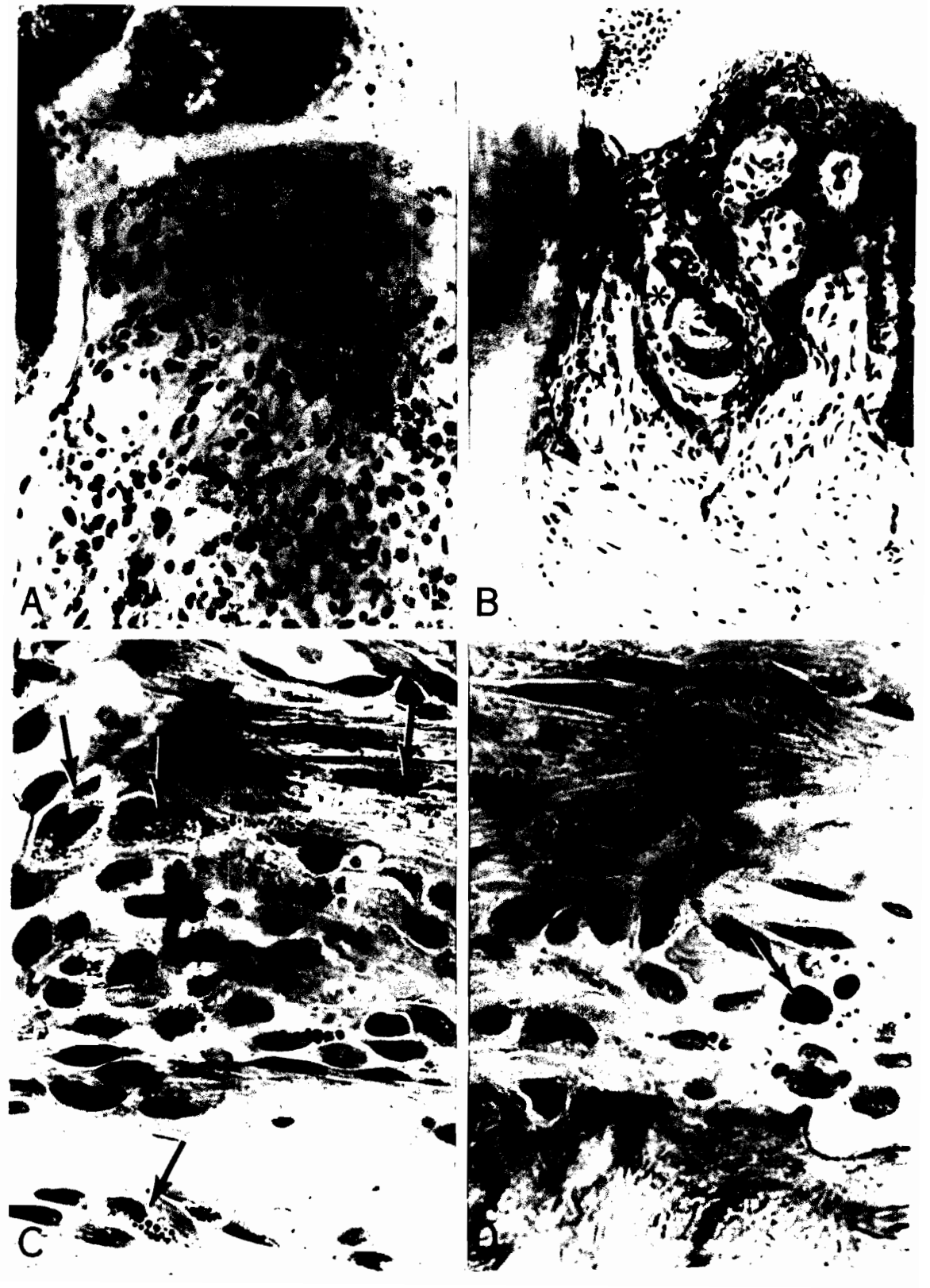
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FIGURE 5 - A-D

Decalcified 3 μm sections of alveolar bone and periodontal ligament in rice rats given Harvard 700 diet for 12 weeks. (A) Low power photomicrograph of alveolar bone of diseased animal at 12 weeks showing highly irregular bone surfaces (Original magnification, 75x). (B) Irregular bone surface of diseased animal with osteoclast on inner aspect of scalloped area (arrow) and replacement of bone with periodontal ligament (Original magnification, 250x). (C) Autoradiography of deeply scalloped bone surface showing ^{3}H-TdR PDL fibroblasts (arrows) adjacent to the bone surface (Original magnification, 250x). (D) Autoradiograph of alveolar crest showing several ^{3}H-TdR labeled cells adjacent to the bone surface (Original magnification, 250x).


D

structure becomes less organized after repeated episodes of resorption and formation.

By 18 weeks of high carbohydrate diet, periodontal disease in the rice rats progresses to severe levels. Figure 6A demonstrates the small amounts of alveolar bone remaining in the interdental area. As tissue destruction continues, the epithelial attachment and subjacent connective tissue often migrate close to the apex of the molar root (Figure 6B). Some animals show almost total destruction of the periodontium, with no functional support of teeth remaining (Figure 6C). The germinative layer of epithelium often shows degenerative change; in some animals the epithelium is denuded entirely, with connective tissue exposed to the oral cavity (Figure 6D). Remaining connective tissue is heavily infiltrated with inflammatory cells. Often, only small spicules of alveolar bone can be observed at 18 weeks. The bone surfaces of some of the spicules appear quiescent, with only PDL fibroblasts present (Figure 7A). Other spicules may contain some osteoclasts (Figure 7B). Normal osseous architecture is completely absent in these remaining fragments of bone.

Quantitative Results

Area based analyses

The effects of the progress of the periodontal syndrome on area based parameters are summarized in Table 2. When compared to animals treated for 6 weeks, per cent bone in M_1 - M_2 interdental area is decreased significantly by approximately 50% at 12 weeks, and 75% at 18 weeks. The per cent vascular spaces is decreased by about 50% and 60%, at 12 and 18 weeks, respectively. The percentage of the test site

FIGURE 6 - A-D

Undecalcified 3 µm sections of the periodontium of rice rats, treated with Harvard 700 diet for 18 weeks. (A) Low power photomicrograph of remaining alveolar bone in severely diseased rice rat (Original magnification 75x). (B) Remaining alveolar bone in rice rat with severe periodontal disease. Note proximity of epithelial attachment (arrow) to apex of molar root (Original magnification 125x). (C) Severe bone loss with only a few remaining bone spicules in the periodontium. Note heavy plaque accumulation present (Original magnification 125x). (D) Higher power photomicrograph of bone spicule seen in Figure 5C with PDL fibroblasts being the cell population at the bone surface (Original magnification 250x).

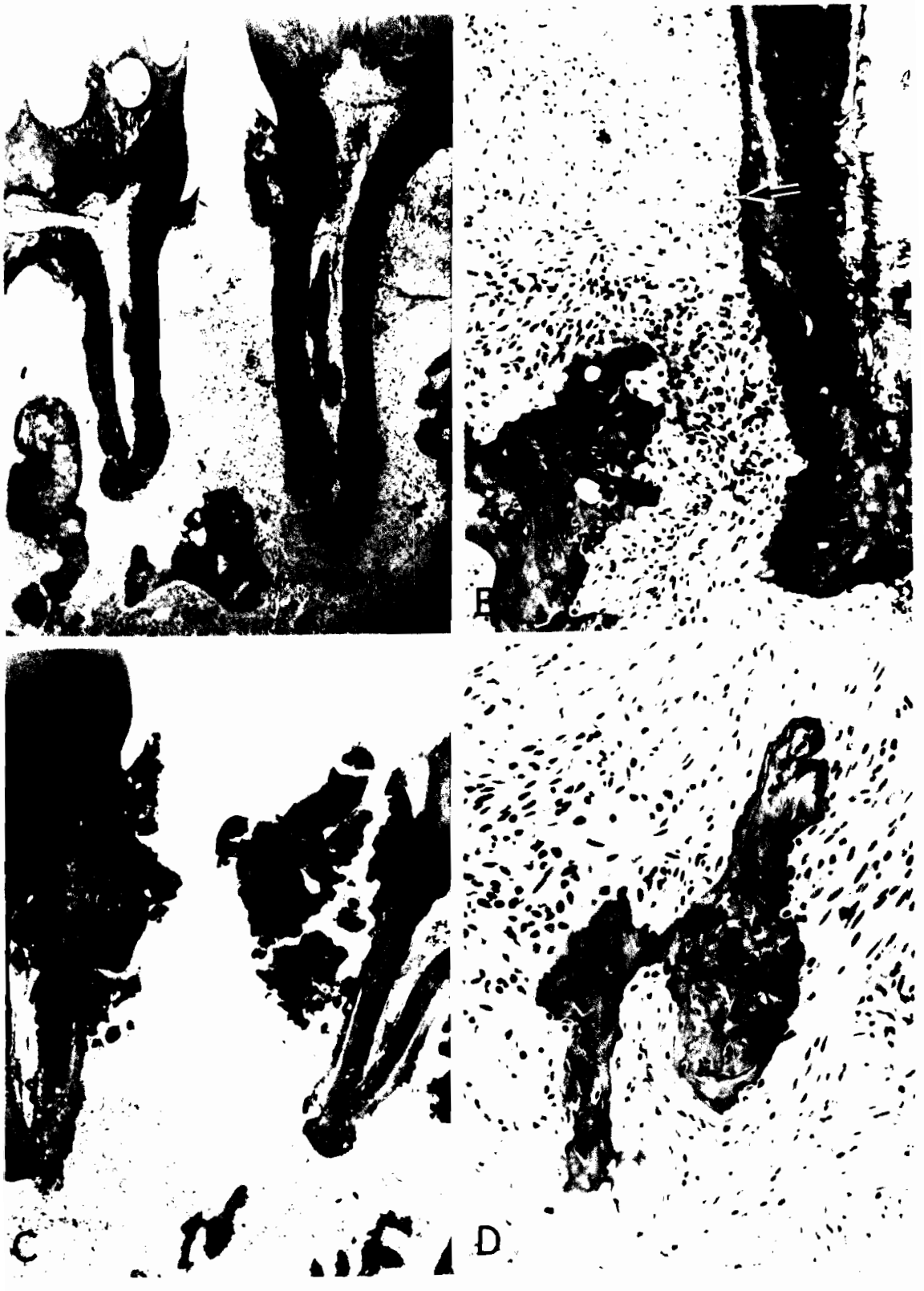
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FIGURE 7 - A-B

Decalcified 3 μm sections from rice rats fed Harvard Ration 700 for 18 weeks. (A) Bone spicules remaining after severe tissue destruction. Note presence of osteoclast (arrow) and many fibroblasts at bone surface (Original magnification 125x). (B) Connective tissue exposed to oral cavity following destruction of epithelium in periodontal disease (Original magnification 250x).

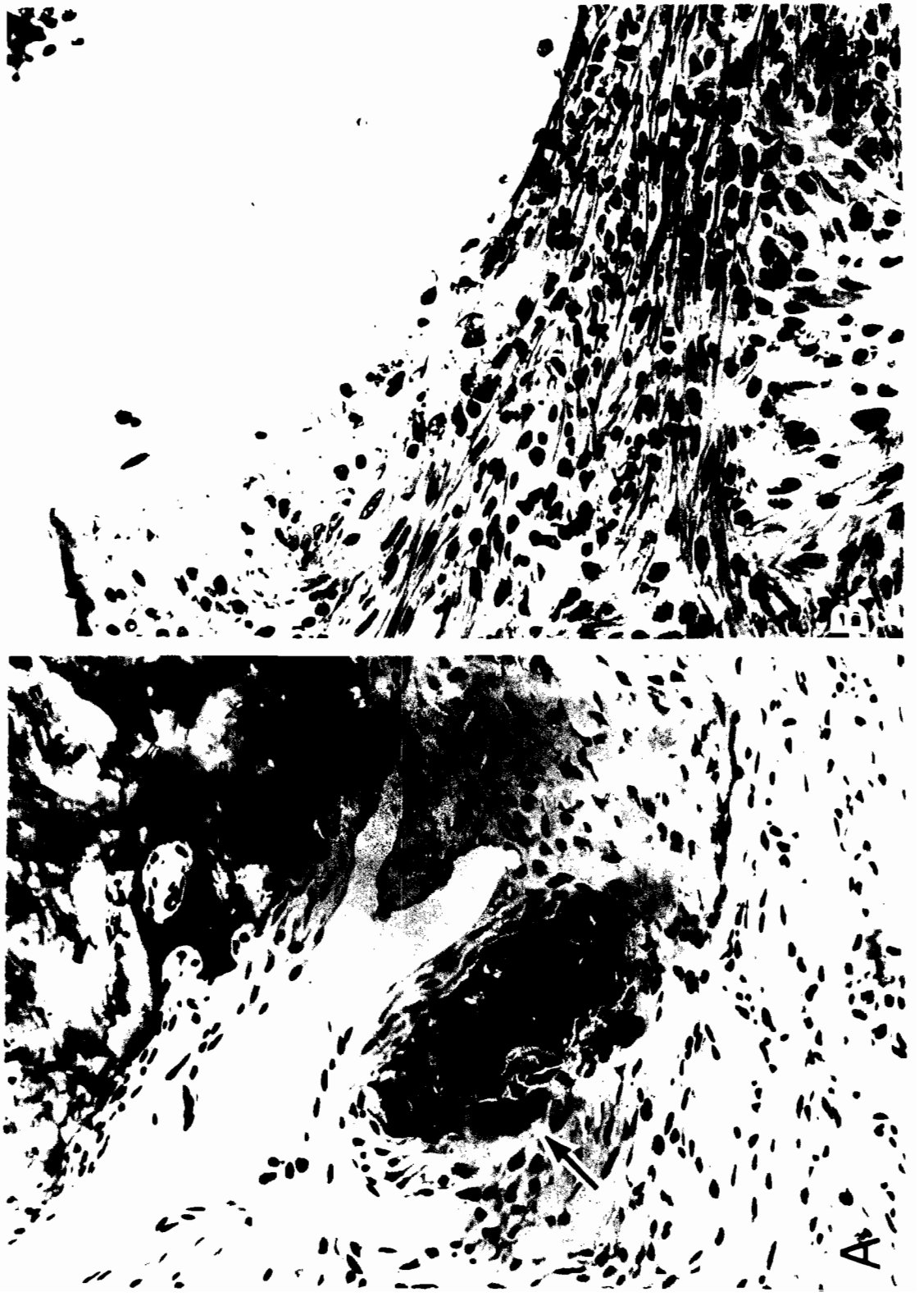
A

TABLE 2

EFFECTS OF THE PERIODONTAL SYNDROME IN THE RICE RAT

ON MORPHOMETRIC AREA PARAMETERS

PERIOD OF TREATMENT	†n	% BONE	% VASCULAR SPACE	% "DESTROYED TISSUE"
6 WEEKS	60	29.2 ±1.3	6.3 ±0.4	3.2 ±0.9
12 WEEKS	24	16.1 ±1.7***	3.2 ±0.9***	16.0 ±1.6***
18 WEEKS	44	8.1 ±1.0***	2.4 ±0.3***	33.5 ±2.9***

Values ± S.E.M. (Standard error of the mean).
†n = Number of Samples: 4 samples per animal.
***p > 0.001, compared to control values.

which has missing areas of tissue increases by 400% at 12 weeks, and by 1000% at 18 weeks.

Cell population analyses.

The summary of cell population data can be seen in Table 3. When compared to 6 weeks, the numbers of periodontal ligament fibroblasts per mm bone surface is not increased at 12 weeks' time; there is, however, a significant increase of 12% at 18 weeks' time. The numbers of osteoblasts is unchanged at any time period. The number of osteoclast nuclei per mm of bone surface is increased significantly by approximately 150%, at both 12 and 18 weeks. The numbers of inflammatory cells per mm surface increases greatly at both 12 and 18 weeks of time. Finally, the mm of bone surface upon which these cell populations reside decreases by approximately 10% and 50%, at 12 and 18 weeks, respectively.

TABLE 3

EFFECT OF PROGRESS OF THE PERIODONTAL SYNDROME IN THE RICE RAT ON CELL POPULATIONS ADJACENT TO THE BONE SURFACE

		CELLS PER MM BONE SURFACE				
PERIOD OF TREATMENT	†n	PDL FIBRO-BLASTS	OSTEO-BLASTS	OSTEO-CLAST NUCLEI	INFLAMMATORY CELLS	mm. BONE SURFACE
6 WEEKS	24	44.92 ±0.95	10.99 ±1.38	2.80 ±0.52	0.19 ±0.11	5.10 ±0.23
12 WEEKS	12	42.45 ±2.17	7.11 ±1.31	6.90 ±1.14***	3.28 ±1.14**	4.71 ±0.46***
18 WEEKS	22	50.13 ±2.00*	8.94 ±1.35	6.47 ±0.69***	3.73 ±0.85**	2.33 ±0.30***

Values ± S.E.M. (Standard error of the mean).
†n = Number of samples: 2 samples per animal.
*p < 0.05
**p < 0.01
***p < 0.005
Compared to control values.

PDL fibroblast proliferation

Changes in PDL fibroblast proliferative activity are summarized in Table 4. The labeling index of PDL fibroblasts is increased by about 100% at both 12 and 18 weeks, as compared to animals treated for 6 weeks. When expressed as the numbers of labeled cells per mm of bone surface, the proliferative activity increases by approximately 100% and 150% at 12 and 18 weeks, respectively.

TABLE 4

EFFECT OF PROGRESS OF THE PERIODONTAL SYNDROME IN THE RICE RAT ON PDL FIBROBLAST PROLIFERATIVE ACTIVITY

PERIOD OF TREATMENT	†n	LABELING INDEX	LABELED CELLS/mm
6 WEEKS	24	0.0165 ±0.0027	0.73 ±0.11
12 WEEKS	12	0.0346 ±0.0109	1.44 ±0.40
18 WEEKS	22	0.0353 ±0.0064*	1.79 ±0.33**

Values ± S.E.M. (Standard error of the mean).
†n = Number of Samples: 2 samples per animal.
*p < 0.05
**p < 0.025
Compared to control values.

DISCUSSION

The most prominent effect of the progression of periodontal disease in the rice rat was a dramatic loss of bone and connective tissue after extended periods of high carbohydrate diet. Bone loss proceeded at a relatively constant rate from 6 through 18 weeks of dietary treatment, with approximately 8% bone loss per week. Using gross anatomical surveys (Gupta & Shaw 1956a) on "prone" animals, Mulvihill et al. (1967) have data indicating a linear rate of bone loss in their rice rat studies. These similarities of amounts and rates of bone loss in different experiments point out the reproducible nature of periodontal disease in the rice rat, and lend further encouragement to use of the rice rat in testing newer treatments to reduce periodontal destruction.

The presence of large vascular spaces within the periodontium of the rice rat has been noted previously (Gupta & Shaw 1956a, Hattler et al. 1977). The present experiment described the change in the amounts of vascular space with time. The data reveals that areas of vascular space decreased with the progression of periodontal disease. Inspection of histological sections revealed that vascular spaces decreased as the connective tissue of the periodontium became fibrotic with increasing severity of periodontal disease. Finally, the amount of tissue destruction in this defined area of the periodontal ligament increased dramatically over time. In this experiment, tissue destruction increased in a linear fashion, as the amount of bone in the interdental area decreased.

The study of cell populations at the bone surface revealed changes in numbers of fibroblasts, osteoclast nuclei, and inflammatory cells. The slight increase in PDL fibroblasts per mm of bone surface is probably due to local increases in fibroblasts within selected areas of alveolar bone (Figure 5B). As bone was destroyed by osteoclast activity, a deeply scalloped channel was formed. Periodontal ligament with a high fibroblast density then replaced the destroyed bone.

It is of interest to note that, although amounts of bone decreased greatly with age in this animal model, the numbers of osteoblasts remained unchanged at any time period sampled. Although many bone surfaces are devoid of osteoblasts at later time periods, there is often a high density of osteoblasts within the highly scalloped bone surfaces described above. These locally increased numbers of osteoblasts may then account for the average numbers of osteoblasts being unchanged with increasing severity of disease.

Increases in osteoclast numbers have been documented in the progression of periodontal disease in the Syrian hamster (Saffar & Baron 1977) and the rice rat (Leonard & Swing 1977). The study of osteoclasts in the hamster showed increases in cell numbers in the lingual periosteal surface and the alveolar (endosteal) surfaces, but not along the bone surfaces adjacent to the periodontal ligament. In the present study, osteoclasts were counted on sagittal rather than horizontal sections. Also, endosteal and periodontal ligament surfaces were combined, as the separation was not consistantly possible in sagittal section. In this study, osteoclast nuclei were quantitated. Dramatic increases in nuclei number were first observed at 12 weeks; these increases were maintained at 18 weeks. As the number of nuclei

per osteoclast did not vary at any time, changes in osteoclast nuclei are proportional to changes in osteoclast numbers. Initially, osteoclasts were found in selected areas along the mesial half of the alveolar bone, where the physiological modeling processes were taking place. As the periodontal disease progressed, osteoclasts were found in a more uniform distribution within the alveolar bone.

The number of inflammatory cells per mm of bone surface also showed dramatic increases by 12 weeks of treatment, and sustained increases at 18 weeks' time. It cannot be determined from the present study what, if any, direct effects these inflammatory cells have on the bone surface upon which they reside. It is known that PMN's, which comprised the majority of the cells counted, have phagocytic properties for a variety of opsonized materials. Also, it has been found that a supernatant from cultured leukocytes is capable of increasing bone resorption *in vitro* (Horton et al. 1972). This substance has been designated as osteoclast activating factor (OAF), and is suggested to stimulate osteoclast activity at a local disease or injury site, independent of any generalized skeletal effect (Horton, Wezeman & Kuettner, 1978). It has been shown recently that OAF is capable of stimulating existing osteoclasts into greater resorptive activity, by increasing the area of the cell, ruffled border zone, and clear zone (Holtrup, Raisz & King 1978). Thus, the PMN's at the bone surface may in part be an additional stimulus for the bone destruction noted here.

Finally, it is of interest to observe the proliferative activity of the PDL fibroblasts near the alveolar bone of the rice rat. The amount of proliferation, whether measured by the labeling index or numbers of labeled cells per mm of bone surface, showed increases at 12

weeks, which were sustained at 18 weeks. These data are in contrast to the labeling index seen in normal aging rats with little or no periodontal inflammation (Stahl, Tonna & Weiss 1969). In studies using aging Sprague Dawley rats, the labeling index of osteogenic cells near the bone surface decreased from about 2% at 8 weeks of age to 0.3% at 18 months of age. The increase in labeling in the rice rat cannot be contributed to an aging phenomenon, but rather a direct consequence of the spreading periodontal inflammation in these animals.

As with the fibroblast and osteoblast populations, the increases seen in ^{3}H-TdR labeled cells are most prominent in the deeply scalloped areas of bone in periodontally diseased animals. Thus, the increase in proliferative activity of the PDL fibroblast, a structural cell and multipotential cell precursor (Melcher & Eastloe 1969), is linked to increases in fibroblast and maintenance of the osteoblast population. Osteoblast numbers did not increase as fibroblast numbers did, implying that the majority of PDL fibroblasts which were labeled with ^{3}H-TdR were renewing the fibroblast population. It cannot be determined from this study whether different subpopulations of the proliferating fibroblast pool contributed exclusively to either osteoblasts and fibroblasts, or whether a common progenitor cell population provided both cell types. It would be of interest to determine the mechanisms by which the fibroblast population replaces the osteoblast population at the bone surface of periodontally diseased animals.

PART II

THE EFFECTS OF A DIPHOSPHONATE, DISODIUM DICHLOROMETHYLENE DIPHOSPHONATE ON THE PERIODONTAL SYNDROME IN THE RICE RAT (*Oryzomys palustris*)

INTRODUCTION

The diphosphonates are compounds which are capable of inhibiting bone resorption (Russel & Smith 1973, Russell & Fleisch 1975). Diphosphonates are analogs of pyrophosphate, which is postulated to be an endogenous regulator of bone metabolism (Russell, Bisaz & Fleisch 1969). Like pyrophosphate, the diphosphonate compounds are capable of inhibiting both crystal growth (Francis 1969) and dissolution of hydroxyapatite (Fleisch, Russell & Francis 1969). The diphosphonates, unlike pyrophosphates, have a P-C-P bonding structure, which is highly resistant to enzymatic degradation. Thus, the effects of diphosphonates are sustained for long periods *in vivo*.

Two diphosphonate compounds have been studied in detail: ethane 1-hydroxy-1, 1-diphosphonate (EHDP), and dichloromethylene diphosphonate (Cl_2MDP). Both have been shown to be effective in several experimental situations, and both are promising as clinical agents to prevent bone loss. Of these two compounds, Cl_2MDP has been shown to be more effective than EHDP, at equivalent doses, in a number of *in vivo* experiments (Russell et al. 1970, Schenk et al. 1973, Miller & Jee 1977). Cl_2MDP inhibits normal bone resorption in the metaphysis of growing rats, so that hard tissue mass in this region increases greatly. The increases in metaphyseal hard tissue mass are proportional to the dose of Cl_2MDP administered to the animal (Miller & Jee 1977). Cl_2MDP, in addition to being more effective than EHDP, does not inhibit mineralization of osteoid, as has been shown for high doses of EHDP, with

experimental animals (King, Francis & Michael 1971, Miller & Jee 1975), and humans (Jowsey et al. 1971). Thus, Cl_2MDP appears to be the more desirable of these two diphosphonates for the prevention of bone resorption.

The effects of diphosphonates are largely untested in cases of bone loss due to periodontal disease. One recent study tested the effects of EHDP on the periodontal bone loss in hamsters fed a high carbohydrate diet (Schaaf, Kafrawy & Standish 1978). Using the gross anatomical survey method of Keyes (Keyes & Gold 1955), there was no effect of EHDP on bone loss. There was, however, an increase in root ankylosis in EHDP treated animals. Effects of Cl_2MDP on experimental periodontal disease have not been reported.

This experiment involved long term treatments of the rice rat (Oryzomys palustris) with graded doses of diphosphonate and a high carbohydrate diet which induced periodontal disease. The periodontal disease of the rice rat involves a rapid loss of bone and connective tissue. The progress of this periodontal destruction has been documented in detail in Part I of this manuscript. The periodontal syndrome in the rice rat involves reproducible changes in morphological, cellular, and proliferative parameters, and these parameters may be used to study the effect of therapeutic agents on the disease process.

The diphosphonate chosen for this study is Cl_2MDP, because its effects are untested in experimental periodontal disease, and because Cl_2MDP has more efficacy and fewer harmful effects than EHDP. The purposes of this experiment are 1) to assess the actions of graded doses of Cl_2MDP upon the progress of the periodontal syndrome in the rice rat, and 2) to assess any Cl_2MDP action on the inflammatory process and

associated connective loss of periodontal disease. The hypotheses tested are 1) that Cl_2MDP would retard or prevent the bone loss of the periodontal syndrome in the rice rat, and 2) that Cl_2MDP would decrease the inflammatory response associated with periodontal disease in this animal model.

MATERIALS AND METHODS

Experimental Design

This experiment used 76 growing rice rats (Oryzomys palustris), of both sexes, which had been selected from our breeding colony, and bred selectively as periodontal disease prone animals. The animals were raised and maintained under identical conditions to those listed in Part I of this manuscript; the animals described in Part I served as controls for this dose response experiment.

The basic design of this experiment is summarized in Table 5. The rice rats were treated for 6, 12 or 18 weeks with the Harvard 700 diet, and with either saline (control) or graded doses of disodium dichloromethylene diphosphonate (Cl_2MDP)*. Animals were incorporated into the experiment at weaning (21 days of age); treatment of animals began at age 22 days. Animals were entered as new weanlings became available. All animals within a litter were distributed equally among treatment groups by random assignment, as animals were weaned.

There were at least 2 animals per group, with each group receiving a single daily subcutaneous injection of solution, in the amount of 1.0 ml per 100 g. body weight. Experimental groups received either 0.9% sodium chloride solution (Bacteriostatic Saline, Abbott Laboratories, N. Chicago, Ill.) as controls, or 0.1, 1.0, or 10.0 mg per kg per day of Cl_2MDP. The Cl_2MDP doses were separated by a factor of 10,

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*The Cl_2MDP was a gift from Procter & Gamble, Cincinnati, Ohio.

TABLE 5

SUMMARY OF EXPERIMENTAL DESIGN OF TREATMENT OF PERIODONTAL DISEASE IN RICE RATS WITH SIMULTANEOUS ADMINISTRATION OF Cl_2MDP

LENGTH OF TIME OF TREATMENT	DOSE OF Cl_2MDP (mg/kg/day)			
	0	0.1	1.0	10.0
6 WEEKS	n = 12	n = 4	n = 5	n = 4
12 WEEKS	n = 6	n = 4	n = 4	n = 6
18 WEEKS	n = 11	n = 8	n = 5	n = 7

n = Numbers of animals per group.

with the lowest dose given being the theoretical lowest effective dose of Cl_2MDP action in the tibial metaphysis of rats, when given for 30 days (Gotcher, Kimmel & Jee 1976). Cl_2MDP solutions were obtained by dissolving the sodium salt of the compound in Bacteriostatic Saline for the 0.1 mg/kg/day dose level, and sterile water (Abbott Laboratories) for the 1.0 and 10.0 mg/kg/day dose levels. The pH of all Cl_2MDP solutions was adjusted to 7.37 by titration with 0.1 N NaOH. Animals were weighed and injected at the same time daily (8:00--9:00 a.m.). The weights and general health of animals treated with Cl_2MDP were monitored to detect any deleterious effect of the drug during these longer treatment periods.

At one hour prior to scheduled autopsy, at the end of assigned treatment periods, each animal was given a single subcutaneous injection of tritiated thymidine (^{3}H-TdR), in identical manner as described in Part I of this manuscript.

Histological Techniques

All tissues were processed exactly as described in Part I, with exceptions noted below.

At autopsy, the tibiae were opened longitudinally into the marrow cavity with a razor blade, then allowed to fix in 10% formalin solution for 24 hours. These bones were dehydrated and defatted with multiple changes of acetone and ether. Tibiae were then embedded in polystyrene resin (FASCO, Inc., Salt Lake City, Utah), and cut in longitudinal sections, at 300 μm intervals, using a precision saw. Sections were compared, and an equivalent single section from each animal was ground and polished to a final thickness of 100 μm. These sections were microradiographed (12 kv, 30 ma) on Kodak 649-0 Spectroscopic plates for study of metaphyseal areas occupied by mineralized tissues (bone and calcified cartilage).

Quantitative Methods

Mandibular analysis

The quantitative analyses of the mandible in the rice rats were identical to those described in Part I; therefore, the parameters studied will be listed below:

1. Area based analyses.
   - A. Per cent bone
   - B. Per cent vascular spaces
   - C. Per cent "destroyed tissue" space
2. Cell population analyses
   - A. PDL fibroblasts per mm bone surface
   - B. Osteoblasts per mm bone surface

C. Osteoclast nuclei per mm bone surface

D. Inflammatory cells per mm bone surface

E. mm of bone surface

3. Proliferative activity of PDL fibroblasts

A. Labeling index of PDL fibroblasts

B. Labeled PDL fibroblasts per mm bone surface

Tibial metaphyseal analysis

In order to compare the response of the alveolar bone to Cl_2MDP in the rice rat to a known, reproducible response of Cl_2MDP on bone, the tibial metaphysis of the animals was studied. Detailed descriptions of Cl_2MDP action on the tibia have been published recently (Miller & Jee 1977); this tissue shows dramatic effects to Cl_2MDP administered for 10 days' time.

The microradiographs of tibial sections were studied using a Quantimet 700 image analyzing computer (QTM; Imanco, Division of Metals Research, Ltd., Cambridge, England). By the use of an image editor pen with the QTM, a reproducible area 5.0 mm distal to the epiphyseal plate, and including only the metaphyseal trabecular bone, could be studied (Figure 8). Using a standard threshold setting, the tibial microradiographs were observed at a microscope magnification of 1x. An internal program calculated the per cent metaphyseal bone (mm^2 of trabecular bone/mm^2 of trabecular bone + marrow in the metaphysis). Using this parameter, changes in bone mass were quantitated rapidly.

Statistical Methods

The treatment of all data was identical to methods described in Part I. In addition, pooled values of per cent bone and per cent

"destroyed tissue" space were plotted against each other, and appropriate regression lines described for each dose level of Cl_2MDP. Significance between these regression lines was tested by analysis of covariance methods (Snedecor & Cochran, 1967).

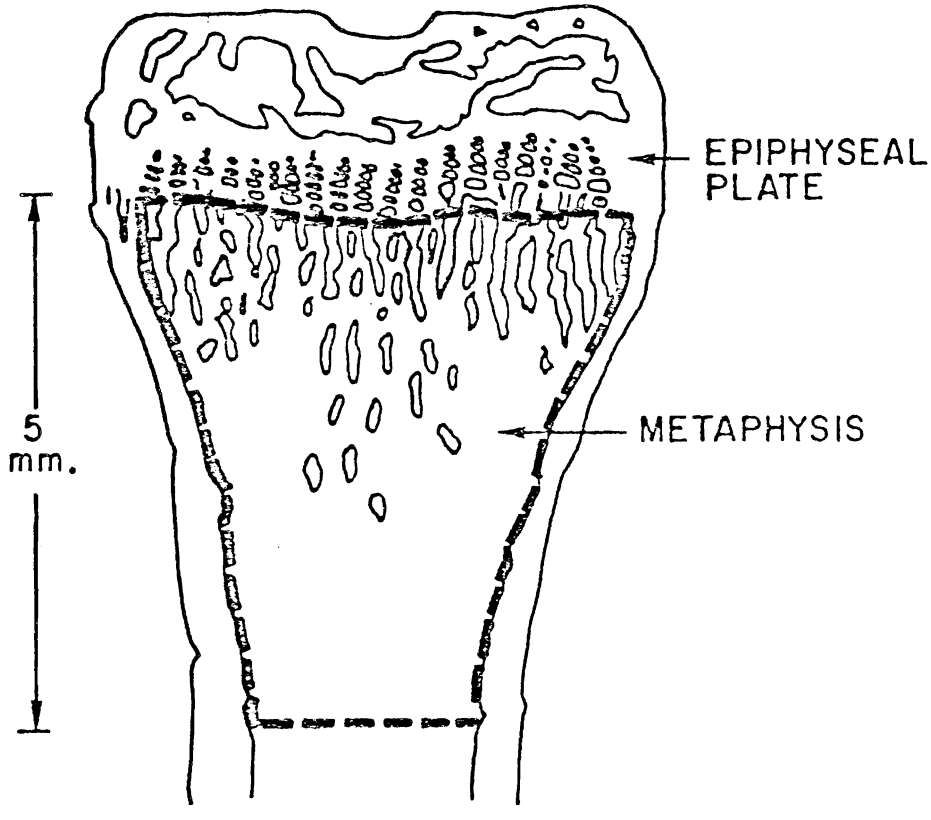


FIGURE 8. Diagram of longitudinal section of tibial metaphysis, showing metaphyseal area studied with the Quantimet 720 within heavy dashed lines.

RESULTS

Observations

Rice rats that are administered Cl_2MDP experience no weight loss, dimunition of growth, or other deleterious effects during the periods of this experiment, in comparison to litter matched controls.

Mandible

As the descriptions of the progress of the periodontal syndrome has been reported in the accompanying manuscript, only the changes associated with Cl_2MDP administration will be reported here.

In animals with little or no periodontal disease, no differences can be observed between age matched controls and treated animals at any dose level. However, when significant tissue destruction takes place, obvious differences are noted between controls and animals treated with 1.0 and 10.0 mg/kg/day of Cl_2MDP, but not at 0.1 mg/kg/day of drug (Figures 9A,B). In diseased animals treated with high doses of Cl_2MDP, trabeculae of alveolar bone are observed protruding into the oral cavity or well into the oral epithelium (Figures 9C,D, 10A,B). This bone is devoid of typical bone cells, and instead is lined by epithelial cells, heavy infiltrations of PMN's, or accumulations of bacterial plaque. In general, the amount of apical epithelial migration and tissue destruction in the 1.0 and 10.0 mg/kg/day dose levels is greater than that of litter matched controls. Remaining bone in treated animals often has an unusual delicate scalloped structure (Figure 10C),

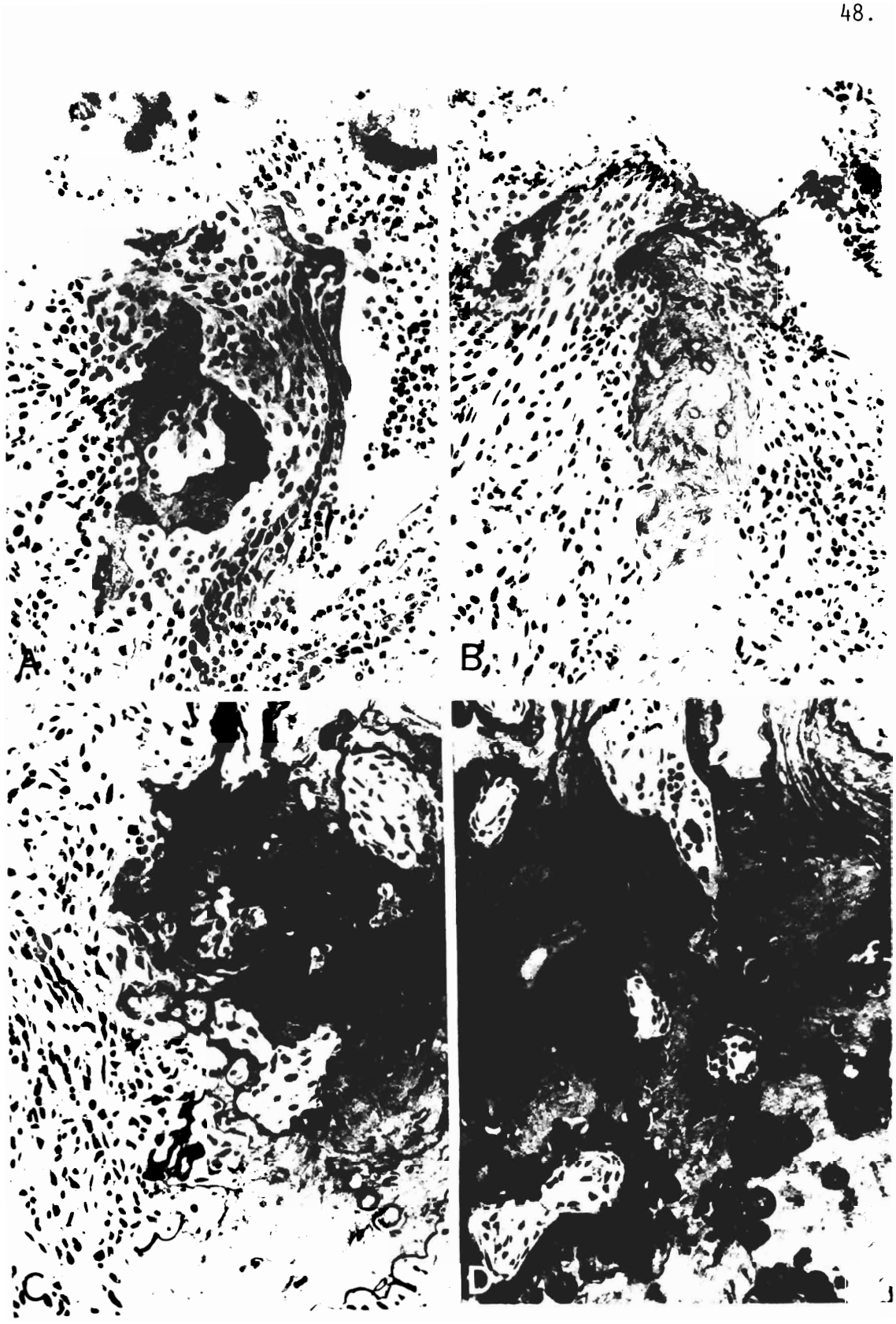
FIGURE 9 - A-D

Decalcified 3 μm sections of rice rats given high carbohydrate diet and Cl_2MDP for 18 weeks. (A) Periodontium of control animal fed Harvard 700 diet for 18 weeks. (B) Animal fed Harvard 700 diet and given 10.0 mg/kg/day of Cl_2MDP. (Both original magnifications 75x). (C,D) Cl_2MDP treated animals, with remaining bone protruding into the oral cavity.


C
D

FIGURE 10 - A-D

Decalcified 3 µm sections of alveolar bone in Cl_2MDP treated rice rats with severe periodontal disease. (A,B) Examples of alveolar bone protruding into the oral cavity or epithelium. (C) Unusual appearance of alveolar bone in Cl_2MDP treated animals. Note extensive scalloping of the bone surface. (Original magnification, 250x). (D) Alveolar bone of rice rat treated with 10.0 mg/kg/day of Cl_2MDP for 18 weeks, H and E stain. The bone has several darkly staining areas of woven bone upon highly scalloped surfaces of the original alveolar bone. (Original magnification, 125x).


A
B
C
D

or darkly staining woven bone formed within these highly scalloped bone surfaces (Figure 10D). Thus, even though there is an equal or greater amount of tissue destruction in animals treated with 1.0 or 10.0 mg/kg/day of Cl_2MDP, there is a greater amount of bone present in these animals.

Tibial Metaphysis

Cl_2MDP has a profound effect on the mineralized tissues of this rapidly growing area of the tibia. At 6 weeks of treatment, there is an increased bone mass noticeable at 1.0 and 10.0 mg/kg/day dose levels (Figures 11A-D). At 12 and 18 weeks, there are increases at all dose levels, as compared to controls (Figures 12A-D, 13-A-D). This added bone in the higher doses is due to a dramatic increase in the extent of the primary spongiosa, which is composed of many small trabeculae. In addition, the metaphysis of rice rats treated with higher doses of Cl_2MDP have a club shape, presumably due to abnormalities in the periosteal funneling process of modeling in long bones (Schenk et al. 1973).

Quantitative Results

Mandible

1. Area Based Analyses.

   A. Per Cent Bone (Table 6).

At six weeks time, the percentage of bone in the inter-dental area is increased by approximately 25%, at the 1.0 and 10.0 mg/kg/day dose levels. At 12 weeks of treatment, there are increases in bone of about 25%-50%, at all dose levels. By 18 weeks time, there are increases of approximately 25% at the 0.1 mg/kg/day dose level, and

FIGURE 11 - A-D

Contact microradiographs of tibial metaphyses of rice rats treated with Cl_2MDP for six weeks. A = control; B = 0.1 mg/kg/day of Cl_2MDP; C = 1.0 mg/kg/day; D = 10.0 mg/kg/day.

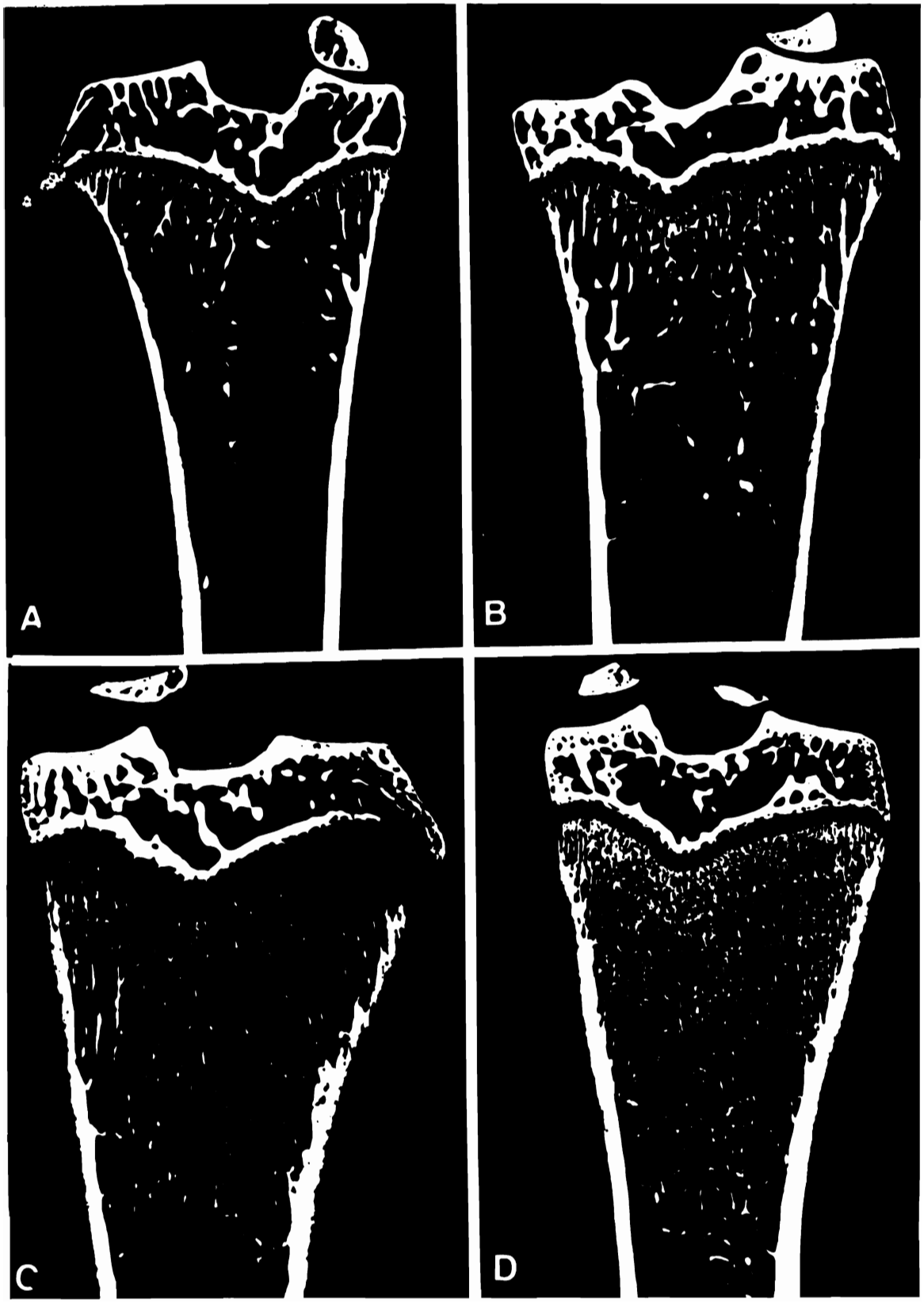

A
B
C
D

FIGURE 12 - A-D

Contact microradiographs of tibial metaphyses of rice rats treated with Cl_2MDP for twelve weeks. A = control; B = 0.1 mg/kg/day of Cl_2MDP; C = 1.0 mg/kg/day; D = 10.0 mg/kg/day.

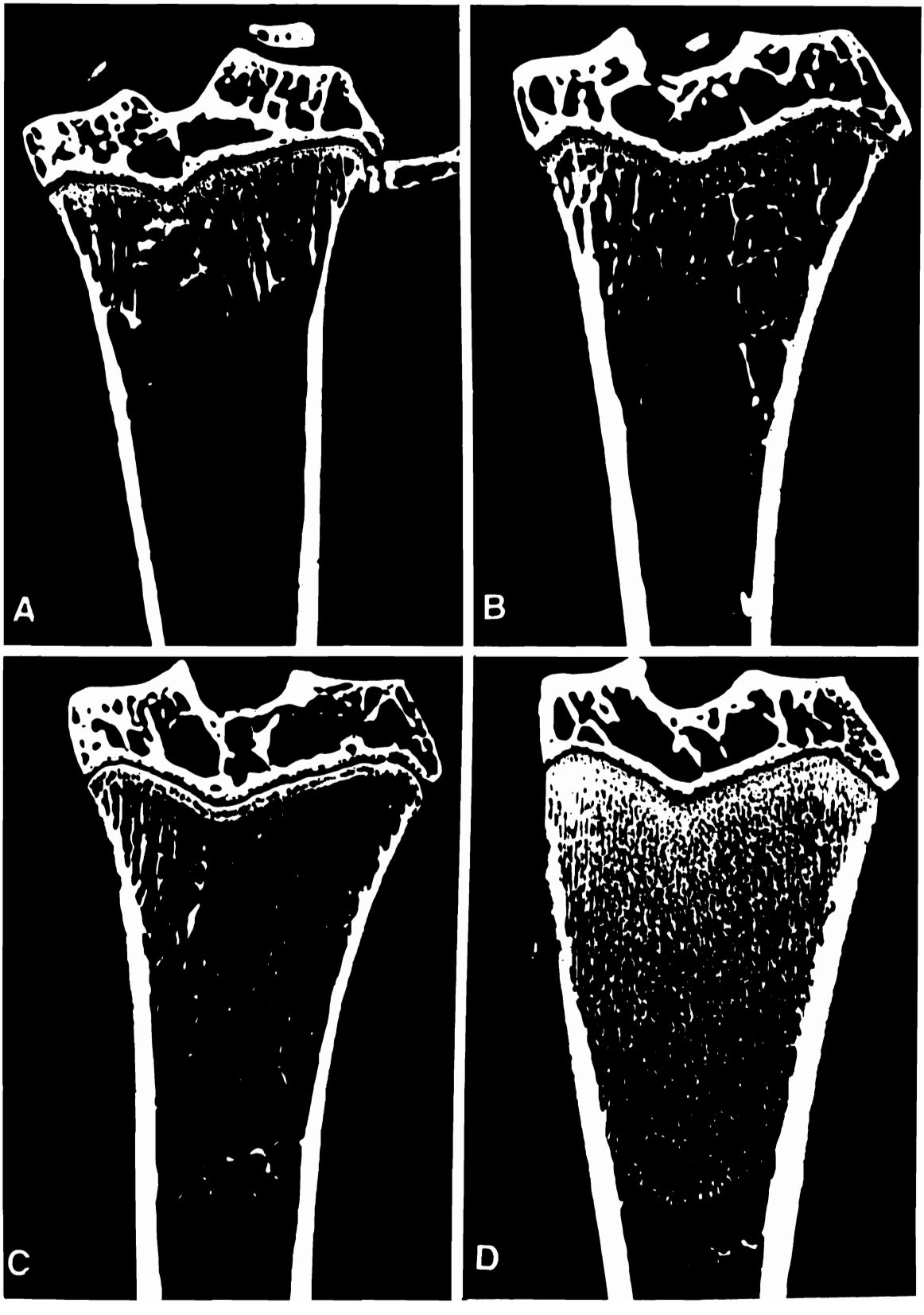

A
B
C
D

FIGURE 13 - A-D

Contact microradiographs of tibial metaphyses of rice rats treated with Cl_2MDP for eighteen weeks. A = control; B = 0.1 mg/kg/day of Cl_2MDP; C = 1.0 mg/kg/day; D = 10.0 mg/kg/day.

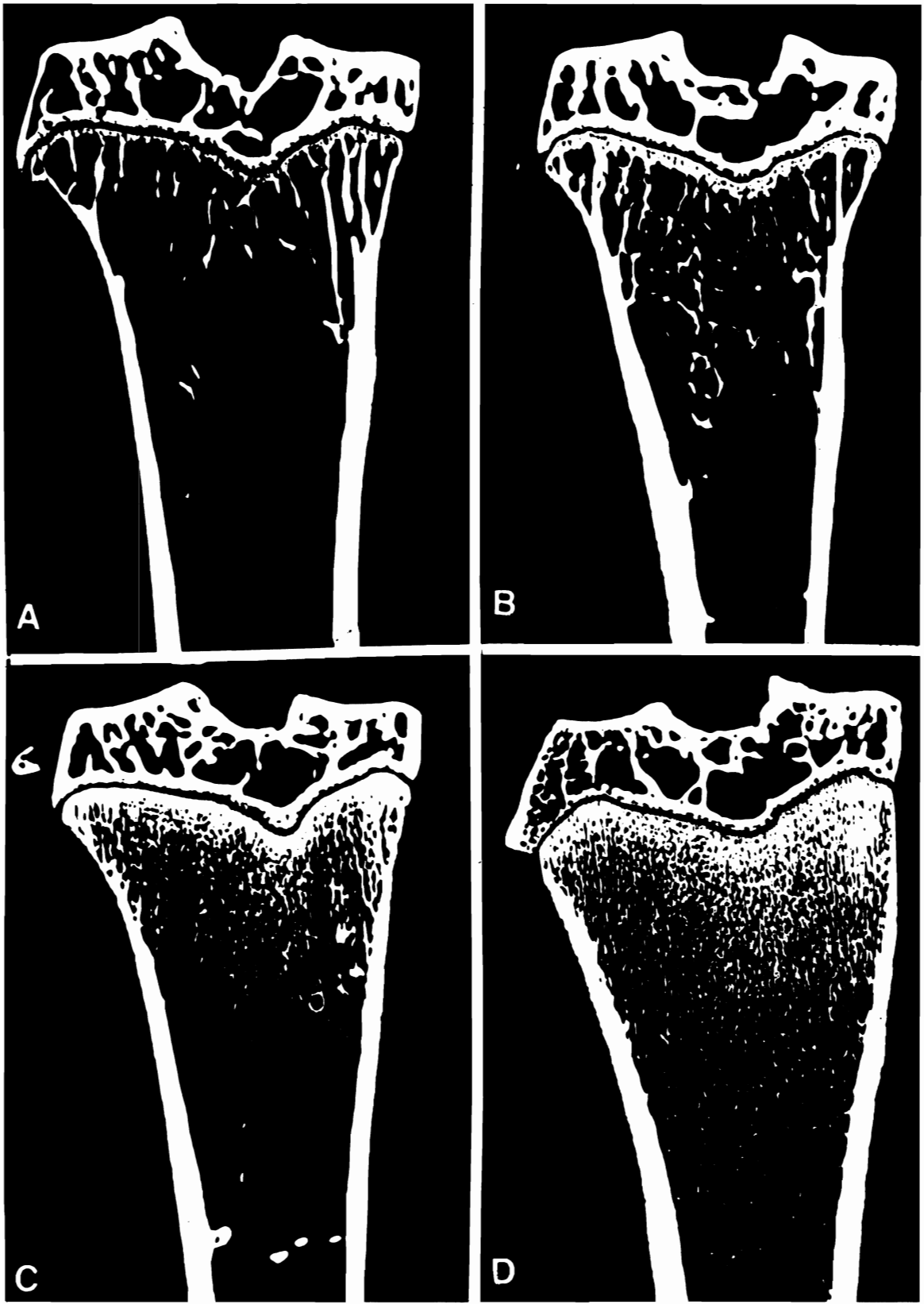

A
B
C
D

TABLE 6

EFFECT OF GRADED DOSES OF Cl_2MDP ON THE PER CENT BONE OF M_1-M_2 INTERDENTAL AREA OF THE RICE RAT

DOSE LEVEL (MG/KG/DAY)	LENGTH OF TREATMENT PERIOD		
	6 WEEKS †(n)	12 WEEKS (n)	18 WEEKS (n)
0	29.2 ±1.3 (60)	16.1 ±1.7 (24)	8.1 ±1.0 (44)
0.1	29.1 ±1.9 (32)	24.0 ±2.8 (16)***	10.7 ±1.7 (32)***
1.0	37.4 ±1.3 (28)***	19.1 ±3.7 (16)***	14.7 ±1.6 (20)***
10.0	35.3 ±2.2 (36)***	23.8 ±2.6 (24)***	15.0 ±0.6 (28)***

Values ± S.E.M. (Standard Error of the Mean).
†n = Number of Samples: 4 samples per animal.
***p > 0.005, compared to control values.

increases of approximately 80% at the 1.0 and 10.0 mg/kg/day doses.

B. Per Cent Vascular Space (Table 7).

Animals treated for 12 weeks with 10.0 mg/kg/day of Cl_2MDP, or animals treated with any dose for 18 weeks show a decrease in the vascularity of the periodontium. Decreases of approximately 65% are seen at 12 weeks, and decreases of about 50% are seen at 18 weeks.

C. Per Cent "Destroyed Tissue" Space (Table 8).

At 6 weeks, there are no significant differences between treated and control animals. At both 12 and 18 weeks, there is a significant increase in tissue destruction, by 75% at 12 weeks, and 15% at 18 weeks, in animals treated with 10.0 mg/kg/day of Cl_2MDP.

TABLE 7

EFFECT OF GRADED DOSES OF Cl_2MDP ON THE PER CENT VASCULAR SPACES IN THE M_1-M_2 INTERDENTAL AREA OF THE RICE RAT

DOSE LEVEL (MG/KG/DAY)	LENGTH OF TREATMENT PERIOD		
	6 WEEKS †(n)	12 WEEKS (n)	18 WEEKS (n)
0	6.4 ±0.4 (60)	3.2 ±0.9 (24)	2.4 ±0.3 (44)
0.1	5.5 ±0.5 (32)	2.6 ±0.6 (16)	1.4 ±0.3 (32)**
1.0	6.8 ±0.5 (28)	2.3 ±0.5 (16)	1.3 ±0.3 (20)**
10.1	5.5 ±0.5 (36)	1.1 ±0.2 (24)**	1.2 ±0.2 (28)**

Values ± S.E.M. (Standard Error of the Mean).
†n = Number of Samples: 4 samples per animal.
**$p < 0.025$, compared to control values.

TABLE 8

EFFECT OF GRADED DOSES OF Cl_2MDP ON PER CENT TISSUE DESTRUCTION WITHIN THE M_1-M_2 INTERDENTAL AREA OF THE RICE RAT

DOSE LEVEL (MG/KG/DAY)	LENGTH OF TREATMENT PERIOD		
	6 WEEKS † (n)	12 WEEKS (n)	18 WEEKS (n)
0	3.2 ±0.9 (60)	16.0 ±1.6 (24)	33.5 ±2.9 (44)
0.1	4.9 ±1.2 (32)	13.3 ±2.9 (16)	27.4 ±2.9 (32)
1.0	0.7 ±0.3 (28)	21.3 ±4.4 (16)	20.7 ±3.1 (20)
10.0	9.8 ±4.6 (36)	28.2 ±3.2 (24)***	37.4 ±4.1 (28)**

Values ± S.E.M. (Standard Error of the Mean)
†n = Number of Samples: 4 samples per animal
**p < 0.25
***p < 0.005
Compared to control values.

D. Per Cent Bone vs. Per Cent Destroyed Tissue Regression Study (Figure 14).

Comparison of regression lines for Per Cent Bone and Per Cent Destroyed Tissue shows that all dose levels have a similar slope, with the 10.0 mg/kg/day dose level significantly separated from other dose regression lines.

2. Cell Population Analyses.

A. Fibroblasts per mm Bone Surface (Table 9).

The number of fibroblasts at the alveolar bone surface is reduced by 25% in the animals treated for 6 weeks. As the periodontal syndrome progresses at 12 and 18 weeks, there are no significant differences between control and treated animals.

B. Osteoblasts per mm Bone Surface (Table 10).

At 6 weeks time, there are no significant differences between control and treated animals at any dose. At 12 and 18 weeks, all dose levels show reductions in the osteoblast population, approaching an 85% reduction at the 10.0 mg/kg/day dose level.

C. Osteoclast Nuclei per mm Bone Surface (Table 11).

There are no significant differences between control and treated animals at any dose, for any time period studied.

D. Inflammatory Cells per mm Bone Surface (Table 12).

There are significant increases in numbers of inflammatory cells at the 10.0 mg/kg/day dose of Cl_2MDP at 6, 12 and 18 weeks.

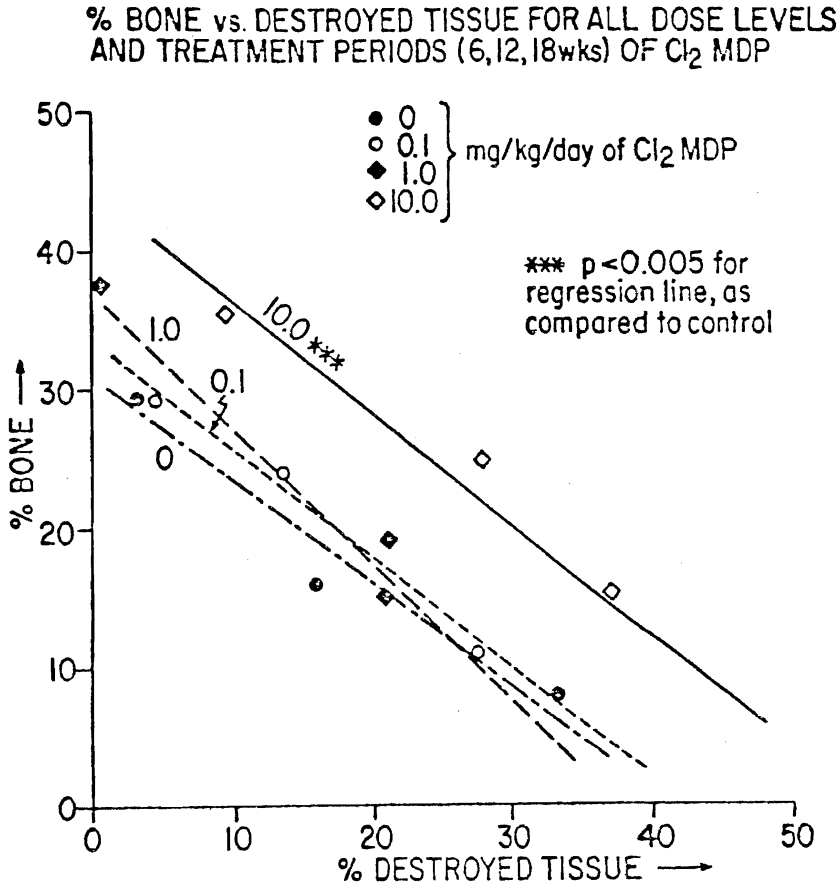


FIGURE 14. Linear regression plots of per cent bone vs. per cent destroyed tissue in the alveolar bone of rice rats treated with Cl_2MDP. All dose levels and time periods of treatment have been plotted in this analysis.

TABLE 9

EFFECT OF Cl_2MDP ON NUMBERS OF FIBROBLASTS PER MM BONE SURFACE IN THE RICE RAT

DOSE LEVEL (MG/KG/DAY)	LENGTH OF TREATMENT PERIOD		
	6 WEEKS †(n)	12 WEEKS (n)	18 WEEKS (n)
0	44.92 ±0.95 (24)	42.45 ±2.17 (12)	50.13 ±2.47 (22)
0.1	44.92 ±2.42 (8)	55.69 ±3.41 (8)	54.73 ±4.29 (16)
1.0	41.03 ±1.32 (10)	56.96 ±3.36 (8)	47.44 ±2.33 (10)
10.0	33.29 ±4.16 (8)***	47.76 ±2.60 (11)	41.59 ±5.02 (14)

Values ± S.E.M. (Standard Error of the Mean).
†n = Number of Samples: 2 samples per animal.
***p < 0.005, compared to control values.

TABLE 10

EFFECT OF Cl_2MDP ON NUMBERS OF OSTEOBLASTS PER MM BONE SURFACE IN THE RICE RAT

DOSE LEVEL (MG/KG/DAY)	LENGTH OF TREATMENT PERIOD		
	6 WEEKS †(n)	12 WEEKS (n)	18 WEEKS (n)
0	10.99 ±1.38 (24)	7.11 ±1.31 (12)	9.94 ±1.67 (22)
0.1	12.74 ±2.66 (8)	3.68 ±0.56 (8)***	3.15 ±0.72 (16)***
1.0	14.00 ±2.20 (10)	2.28 ±0.57 (8)***	3.70 ±0.81 (10)***
10.0	10.10 ±2.97 (8)	1.02 ±0.32 (10)***	1.81 ±0.41 (14)***

Values S.E.M. (Standard Error of the Mean)
†n = Number of Samples: 2 samples per animal.
***p < 0.005, compared to control values.

TABLE 11

EFFECT OF Cl_2MDP ON THE NUMBER OF OSTEOCLAST NUCLEI PER MM BONE SURFACE IN THE RICE RAT

DOSE LEVEL (MG/KG/DAY)	LENGTH OF TREATMENT PERIOD					
	6 WEEKS	†(n)	12 WEEKS	(n)	18 WEEKS	(n)
0	2.80 ±0.52	(24)	6.90 ±1.14	(12)	6.47 ±0.69	(22)
0.1	5.05 ±1.92	(8)	4.75 ±1.04	(8)	6.69 ±1.22	(16)
1.0	4.23 ±0.84	(8)	6.08 ±1.83	(8)	5.10 ±0.83	(10)
10.0	4.23 ±1.55	(8)	4.56 ±0.57	(11)	3.78 ±0.86	(14)

Values S.E.M. (Standard Error of the Mean).
†n = Number of Samples: 2 samples per animal.

TABLE 12

EFFECT OF Cl_2MDP ON NUMBERS OF INFLAMMATORY CELLS PER MM BONE SURFACE IN THE RICE RAT

DOSE LEVEL (MG/KG/DAY)	LENGTH OF TREATMENT PERIOD					
	6 WEEKS	†(n)	12 WEEKS	(n)	18 WEEKS	(n)
0	0.19 ±0.11	(24)	3.28 ±1.14	(12)	3.73 ±0.85	(22)
0.1	1.92 ±1.14	(8)	3.34 ±1.67	(8)	2.69 ±1.17	(16)
1.0	0.54 ±0.42	(10)	7.85 ±2.85	(8)	1.25 ±0.44	(10)
10.0	13.60 ±8.94	(8)**	13.99 ±3.97	(11)**	8.62 ±2.70	(14)**

Values ± S.E.M. (Standard Error of the Mean).
†n = Number of Samples: 2 samples per animal.
**$p < 0.025$, compared to control values.

E. Bone Surface in mm (Table 13).

At 6 weeks, there are no differences in the amount of bone surface between any groups. At 12 weeks, the 10.0 mg/kg/day dose shows a 25% increase over controls. At 18 weeks, both the 1.0 and 10.0 mg/kg/day dosages show significant increases of approximately 90% in mm of bone surface.

3. Proliferative Activity of PDL Fibroblasts (Tables 14, 15).

At all time periods, the 10.0 mg/kg/day dose of Cl_2MDP causes a reduction in the labeling index of PDL fibroblasts, by 94%, 84%, and 64% at 6, 12 and 18 weeks, respectively. When expressed as labeled PDL fibroblasts per mm of bone surface, corresponding decreases in proliferative activity are seen at the 10.0 mg/kg/day dose level of Cl_2MDP.

TABLE 13

EFFECT OF Cl_2MDP ON MM OF BONE SURFACE IN THE RICE RAT

DOSE LEVEL (MG/KG/DAY)	LENGTH OF TREATMENT PERIOD					
	6 WEEKS	†(n)	12 WEEKS	(n)	18 WEEKS	(n)
0	5.10 ±0.23	(24)	4.71 ±0.46	(12)	2.33 ±0.30	(22)
0.1	4.76 ±0.19	(8)	4.66 ±0.53	(8)	2.53 ±0.30	(16)
1.0	4.93 ±0.35	(10)	3.84 ±0.59	(8)	4.49 ±0.51	(10)***
10.0	3.86 ±0.56	(8)	5.92 ±0.46	(11)*	4.35 ±0.44	(14)***

Values ± S.E.M. (Standard Error of the Mean).
†n = Number of Samples: 2 samples per animal.
*p < 0.05
***p < 0.005
Compared to control values.

TABLE 14

EFFECT OF Cl_2MDP ON THE LABELING INDEX OF PDL FIBROBLASTS IN THE RICE RAT

DOSE LEVEL (MG/KG/DAY)	LENGTH OF TREATMENT PERIOD					
	6 WEEKS	†(n)	12 WEEKS	(n)	18 WEEKS	(n)
0	0.0165 ±0.0027	(24)	0.0348 ±0.0109	(12)	0.0353 ±0.0064	(22)
0.1	0.0191 ±0.0046	(8)	0.0129 ±0.0045	(8)	0.0141 ±0.0036	(16)
1.0	0.0123 ±0.0025	(10)	0.0256 ±0.0052	(8)	0.0253 ±0.0064	(10)
10.0	0.0010 ±0.0010	(8)***	0.0057 ±0.0020	(12)*	0.0126 ±0.0060	(14)***

Values ± S.E.M. (Standard error of the mean).
†n = Number of Samples: 2 samples per animal.
*p < 0.05
**p < 0.025
***p < 0.005
Compared to control values.

TABLE 15

EFFECT OF Cl_2MDP ON THE NUMBERS OF LABELED PDL FIBROBLASTS PER MM IN THE RICE RAT

DOSE LEVEL (MG/KG/DAY)	LENGTH OF TREATMENT PERIOD		
	6 WEEKS †(n)	12 WEEKS (n)	18 WEEKS (n)
0	0.73 ±0.11 (24)	1.44 ±0.40 (12)	1.79 ±0.33 (22)
0.1	0.83 ±0.19 (8)	0.76 ±0.27 (8)	0.83 ±0.25 (16)
1.0	0.52 ±0.11 (10)	1.47 ±0.30 (8)	1.26 ±0.33 (10)
10.0	0.04 ±0.04 (8)***	0.26 ±0.09 (11)*	0.52 ±0.23 (14)**

Values ± S.E.M. (Standard Error of the Mean).
†n = Number of Samples: 2 samples per animal.
*p < 0.05
**p < 0.025
***p < 0.005
Compared to control values.

Tibial Metaphysis (Table 16).

At all time periods, the 1.0 and 10.0 mg/kg/day dose levels produce a greater quantity of mineralized tissue in the tibial metaphysis. In addition, increases in mineralized tissue are seen at the 0.1 mg/kg/day dose at 12 and 18 weeks time. The increases in quantity are 85% and 170% for 1.0 and 10.0 mg/kg/day doses, at 6 weeks. At 12 weeks, there are increases of 15% at the 0.1 mg/kg/day dose, and increases of 60% and 240%, with the 1.0 and 10.0 mg/kg/day doses, respectively. By 18 weeks time, there are increases of 30%, 100%, and 270%, at the respective doses of 0.1, 1.0, and 10.0 mg/kg/day of Cl_2MDP.

TABLE 16

EFFECT OF Cl_2MDP IN GRADED DOSES ON THE MINERALIZED TISSUE OF THE TIBIAL METAPHYSIS AT 6, 12, AND 18 WEEKS OF TREATMENT IN THE RICE RAT

DOSE LEVEL (MG/KG/DAY)	LENGTH OF TREATMENT PERIOD		
	6 WEEKS †(n)	12 WEEKS (n)	18 WEEKS (n)
0	14.41 ±1.05 (15)	14.11 ±1.01 (14)	13.32 ±0.97 (14)
0.1	14.21 ±1.31 (9)	16.38 ±1.29 (8)***	17.28 ±1.49 (10)***
1.0	26.66 ±4.76 (7)***	22.64 ±2.66 (7)***	27.29 ±1.67 (10)***
10.0	38.54 ±3.54 (10)***	47.50 ±2.55 (9)***	49.14 ±2.94 (6)***

Values ± S.E.M. (Standard Error of the Mean).
†n = Number of Samples: 1 sample per animal.
***p < 0.001, compared to control values.

DISCUSSION

The main effect of Cl_2MDP in this experiment was to increase the bone mass in the interdental alveolar bone. These findings were in agreement with several experiments where normal or pathological bone resorption has been inhibited or retarded by diphosphonate administration. The increases in bone mass seen after 6 weeks of treatment were probably due to inhibition of bone resorption in the final modeling of the alveolar bone to mature size. The increases seen at 12 and 18 weeks, however, were likely due to a partial inhibition of bone resorption secondary to severe periodontal disease.

It is of interest to compare the effects of Cl_2MDP and fluoride in preserving bone mass in experimental periodontal disease. There is evidence that dietary fluoride may preserve bone mass in golden hamsters (Costich et al. 1957), but not in Wistar Rats (Kristofferson, Bang & Meyer 1970). In rice rats receiving concomitant doses of 15 or 30 ppm of fluoride with a high carbohydrate diet, there were no reductions in the periodontal destruction of bone, as measured by gross anatomical surveys (Auskaps, Gupta & Shaw 1957). The authors stated that alveolar bone lesions in the rice rat could progress rapidly in almost complete absence of damage to the epithelium or overlying connective tissue. In our experiment, however, bone destruction was retarded in spite of extensive epithelial and connective tissue destruction. Finally, Cl_2MDP

treatments in our experiment did not completely inhibit bone resorption, but rather, retarded resorption so that more bone mass was finally present in Cl_2MDP treated animals.

This experiment demonstrated further differences between EHDP and Cl_2MDP in prevention of bone loss. In a recent experiment (Schaaf, Kafrawy & Standish 1978), hamsters were injected with 10 mg/kg/day of EHDP for periods up to six months. EHDP did not inhibit alveolar bone resorption, but instead caused an ankylosis of molar teeth. In the experiment reported here, Cl_2MDP did retard bone resorption, as measured with morphometric means. In addition, there was no root ankylosis noted at any time period or Cl_2MCP dose level. Thus, Cl_2MDP appears to be more effective than EHDP at prevention of alveolar bone loss, with few morphological alterations of periodontal tissues. These results are analagous to the differing effects of EHDP and Cl_2MDP on growing rat tibiae, where Cl_2MDP is more effective than EHDP. EHDP also induces a morphological alteration of the epiphyseal plate, causing a rachitic appearance, where Cl_2MDP does not alter morphology (Schenk et al. 1973, Miller & Jee 1977).

The mechanisms responsible for the unusual bone morphology seen in diseased animals treated with Cl_2MDP is not known at present. The persistence of highly scalloped spicules of bone imply that, while some bone resorption was occurring, the Cl_2MDP altered bone was unable to be resorbed completely. In addition, the periodontal morphology was altered in animals treated with 1.0 and 10.0 mg/kg/day of Cl_2MDP, with bone protruding well into the oral cavity. The segments of alveolar bone adjacent to the oral cavity were contiguous with remaining interdental alveolar bone, and thus could not be considered as

sequestrae. This alveolar bone appearance has been noted in cases of advanced periodontal disease in humans (Frank & Voegel 1978). These authors presented ultrastructural evidence that these bacteria were not artifactual and that they were capable of minor bone resorption along the bacteria - bone interface.

The decreased vascularity seen in Cl_2MDP treated animals may be due to a direct effect of diphosphonates on vascular proliferation, although there is no data to support or refute this hypothesis. More likely is the possibility that the increased connective tissue destruction and fibrosis of remaining tissue have resulted in a decrease in the vascularity within the defined test area.

The increase in the amount of destroyed tissue within the test area is interesting in view of a recent study of diphosphonate effects in adjuvant-induced arthritis (Francis, Flora & King 1972). Rats were treated with Freund's Adjuvant, then daily doses of EHDP. At doses of 4 mg/kg/day, there was a marked reduction in bone resorption and inflammatory response in this model. The authors speculated that reduced bone resorption, which reduced local tissue calcium and phosphate concentrations, might be indirectly responsible for the reduced inflammation. However, in our study of Cl_2MDP treated rice rats, there was an increase in tissue destruction and inflammation with 10.0 mg/kg/day of diphosphonate. Whether the differences in inflammatory response are due to differences between the diphosphonates used or the animal models used, is not known. The regression plots of per cent bone versus per cent destroyed tissue (Figure 14) point out that, regardless of the amount of tissue destruction, there was a significantly greater amount of bone present in animals given 10.0 mg/kg/day of

Cl_2,MDP. Thus, in the rice rat model, the decrease in bone resorption was not associated with a decreased inflammatory response.

As a result of Cl_2MDP treatment, there were significant changes in several cell populations in the rice rat. The numbers of fibroblasts residing near the bone surface were reduced with the 10.0 mg/kg/day dose, at the 6 week time period only. This reduction at early time periods, but not at later times, can be explained by comparing the control rice rat response for numbers of fibroblasts. Because the numbers of fibroblasts increase with increasing severity of periodontal disease, this control phenomenon could be compensating the fibroblast reductions due to Cl_2MDP. The reduction in fibroblast numbers is most likely due to reductions in the proliferative activity at 10.0 mg/kg/day of Cl_2MDP. Likewise, the numbers of osteoblasts per mm of bone surface showed dramatic decreases at 12 and 18 weeks. These decreases were apparent at all Cl_2MDP doses, indicating that osteoblast populations were much more sensitive to the drug than were the fibroblast populations.

These decreases in osteoblast populations have been reported previously in the proximal tibial metaphysis with EHDP (Miller & Jee 1975) and Cl_2MDP (Miller & Jee 1977); in stimulated periodontal ligament adjacent to alveolar bone, with EHDP (Yee 1976), and in canine ribs (Cabenela & Jowsey 1974) and osteoporotic human patients (Jowsey et al. 1970), both treated with EHDP. The lowest doses in our study (0.1 mg/kg/day) showed a depressive effect on osteoblast populations. This dose was lower than the 0.4 mg/kg/day Cl_2MDP dose found by Miller and Jee, and suggests that Cl_2MDP effects on cell populations are cumulative with extended treatment times.

As Cl_2MDP has been reported to increase the numbers of osteoclasts and the numbers of nuclei per osteoclast (Schenk et al. 1973, Miller & Jee 1977), the findings of no change in osteoclast nuclei numbers at any dose of diphosphonate in the rice rat is of interest. Reasons for the differences in these studies are not known; it is possible that the animal model studied accounts for the differences. Another explanation is that increased numbers of osteoclast nuclei are a transient effect of diphosphonate treatment over shorter periods of time.

The numbers of inflammatory cells adjacent to the bone surface also showed increases with 10.0 mg/kg/day of Cl_2MDP. This increase in PMN's against the bone surface may be due to a direct effect of diphosphonate on these cells, but more likely was due to the progression of soft tissue inflammation and destruction, while the bone surface remained intact. Thus, inflammatory cells were found against the bone surface that would normally be absent without Cl_2MDP treatment.

The effects of Cl_2MDP on the proliferative activity of PDL fibroblasts were compatible with Cl_2MDP effects in the tibial metaphysis (Miller & Jee 1977) and EHDP effects in the tibia (Miller & Jee 1975), and orthodontically stimulated alveolar bone (Yee 1976). The control labeling index in the rice rat is lower than either the tibial metaphysis or the stimulated PDL, yet sizable decreases are seen in all studies following diphosphonate treatment. According to Yee (1976), EHDP treatments reduce not only the proliferation of PDL fibroblasts, but also the differentiation of these cells into osteoblasts. Findings of decreased labeling of PDL fibroblasts and reduced

osteoblast numbers in the rice rat are in agreement with the data for EHDP in the stimulated PDL.

The proximal tibiae of the rice rats used in this study were analyzed for changes in bone mass, and compared to changes in alveolar bone mass. Comparing the responses of each tissue to Cl_2MDP, it became apparent that both tissues responded in like manner to doses of Cl_2MDP, over all time periods. Differences in the two tissues involved the amounts of increases in bone mass. For any given dose, the tibia responded to a greater degree than did the alveolar bone. It appears that the diphosphonate's ability to preserve bone depends upon how rapidly the bone is lost in the test system. Thus, in bone with relatively low amounts of turnover, diphosphonates can be predicted to have minimal effect. It must be pointed out that differences in response between the tibia and alveolar bone may be due in part to different stimuli for resorption: physiological forces in the tibia versus pathological factors in the alveolar bone of the rice rat.

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